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**The effects of ultraviolet B radiation and high temperature on stomal aperture and development in arabidopsis thaliana**

# THE EFFECTS OF ULTRAVIOLET B RADIATION AND HIGH TEMPERATURE ON STOMATAL APERTURE AND DEVELOPMENT IN *ARABIDOPSIS THALIANA*

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## ABSTRACT

Stomata are microscopic pores in the epidermis of plant leaves which allow gas and water vapour exchange between internal plant tissues and the atmosphere. Environmental signals influence both stomatal pore aperture and the number of stomata developed to ensure plants possess the necessary means to effectively manage photosynthesis, water status and leaf cooling. High temperature and ultraviolet-B (UV-B) light can occur simultaneously and have significant effects on stomatal aperture and development, but their combined effects on stomata have not been explored. Epidermal peels and whole leaves of *Arabidopsis thaliana* were used to elucidate the combined effect of these two major environmental signals on stomatal aperture and development and investigate potential signalling pathways. UV-B antagonised the high temperature-induced stomatal opening in wild-type (WT) Arabidopsis plants. No UV-B-induced stomatal closure was observed in mutants deficient in the UV-B photoreceptor, ULTRAVIOLET B RESISTANCE LOCUS 8 (UVR8). Nitric oxide (NO) levels increased in response to UV-B and correlated with stomatal closure, in WT plants, but not in *uvr8* mutants. These results suggest that UV-B antagonises high temperature-induced stomatal opening in a UVR8- and NO-dependent manner. High temperature decreased stomatal density in both WT and *uvr8* mutant plants. UV-B induced a significant reduction in stomatal density in the *uvr8* mutant but not in WT controls at both temperatures tested. These results may suggest that stomatal density is increased by a UVR8-dependent mechanism in response to UV-B but decreased in a UVR8-independent manner. Limited high temperature and UV-B-mediated effects on stomatal index were observed in this study.

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## Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:..... DATE:.....

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## List of abbreviations

| | |
|---|---|
| ABA | Abscisic acid |
| ANOVA | Analysis of variance |
| ARF6 | AUXIN REGULATING FACTOR 6 |
| Arg | Arginine |
| ATP | Adenosine triphosphate |
| BAP module | BZR-ARF-PIF module |
| bHLH | Basic helix-loop-helix |
| BLUS1 | BLUE LIGHT SIGNALING 1 |
| BR | Brassinosteroids |
| BZR1 | BRASSINOSTEROID SIGNALLING POSITIVE REGULATOR 1 |
| CFCs | Chlorofluorocarbons |
| Col-0 | Columbia-0 accession of *Arabidopsis thaliana* |
| $CO_2$ | Carbon dioxide |
| COP1 | CONSTITUTIVELY PHOTOMORPHOGENIC 1 |
| CPDs | Cyclobutene pyrimidine dimers |
| cry1/2 | cryptochrome1/2 |
| DAF-FM-DA | 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate |
| DELLA | Inhibitors of GA (Aspartate-Glutamate-Leucine-Leucine-Alanine motif) |
| DNA | Deoxyribonucleic acid |
| EC | Evening complex; ELF3/4, LUX |
| ELF3/4 | EARLY FLOWERING 3/4; part of EC |
| FMA | FAMA |
| FDA | Fluorescein diacetate |
| GA | Gibberellic acid |
| GC(s) | Guard cell(s) |
| GMC | Guard mother cell |
| Hsp | Heat shock protein |

| | |
|---|---|
| HYH | ELONGATED HYPOCOTYL 5 HOMOLOG |
| HY5 | ELONGATED HYPOCOTYL 5 |
| $H_2O_2$ | Hydrogen peroxide |
| $H^+$ ATPase | Proton pump |
| KCl | Potassium chloride |
| Ler | *Landsberg erecta* accession of *Arabidopsis thaliana* |
| LUX | LUX ARRYTHMO |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| MMC | Meristemoid mother cell |
| MS | Murashige and Skoog |
| MUTE | Regulator of stomatal formation |
| NCED | 9-*cis*-epoxycarotenoid dioxygenase |
| NO | Nitric oxide |
| PAR | Photosynthetically active radiation |
| phot1/2 | phototropin 1/2 |
| phy(B) | phytochrome(B) |
| PIF4 | PHYTOCHROME INTERACTING FACTOR 4 |
| PM | Plasma membrane |
| PP1 | Type 1 protein phosphatase |
| R:FR | Red light to far-red light ratio |
| RNA | Ribonucleic acid |
| R | Red light |
| RUP1/2 | REPRESSOR OF UV-B PHOTOMORPHOGENESIS1/2 |
| SAUR | SMALL AUXIN UP RNA |
| SCRM(2) | SCREAM(2) |
| *SDD1* | STOMATAL DENSITY AND DISTRIBUTION1 |
| SE | Standard error |
| SPCH | SPEECHLESS |
| *TAA1* | *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* |

| | |
|---|---|
| *TMM* | *TOO MANY MOUTHS* |
| Trp | Tryptophan |
| UV | Ultraviolet |
| UV-A | Ultraviolet A light; 315-400nm |
| UV-B | Ultraviolet B light; 280-315nm |
| UV-C | Ultraviolet C light; 100-280nm |
| UVR8 | ULTRAVIOLET B RESISTANCE LOCUS 8 |
| *uvr8-6* | ultraviolet B resistance locus 8 mutant, Col-0 background |
| Ws | Wassilewskija accession of *Arabidopsis thaliana* |
| WT | wild-type |
| *YDA* | *YODA* |
| *YUC8* | Gene coding for flavin monooxygenases |

# CHAPTER 1: INTRODUCTION

As sessile organisms, plants must withstand fluctuating conditions in nature in order to survive, be productive and reproduce. These fluctuations include alterations in temperature, light quality, water and nutrient availability. Plants that have evolved in a specific locale have evolved adaptations to promote their survival in the fluctuating conditions of that environment. They accomplish this by first perceiving environmental signals, then initiating signalling cascades which can regulate a variety of processes. These include altered growth patterns and morphology to optimise sun exposure, protection from cold or heat, production of secondary metabolites to ward off attacks by herbivores, and regulation of internal water status.

When a plant cell perceives a signal, such as light, signalling cascades are initiated, and an appropriate response to the initial signal is produced (Xu et al., 2015). In any one isolated signalling pathway there may be many signalling components preceding the ultimate effector(s), allowing for extensive regulation and adjustment of responses. Plants can perceive and respond to multiple environmental signals simultaneously which promotes survival in a dynamic environment. Extensive interactions and crosstalk between signalling pathways allows fine-tuning of plant responses to changes in the environment. Two environmental signals which show extensive crosstalk are light and temperature (Franklin et al., 2014; Casal and Questa, 2017).

## 1.1 Stomata

A key feature of plants which facilitated life on land was the evolution of a waxy cuticle on the outside of leaves and stems, to protect plants from desiccation (Chater et al., 2017). Plants also needed to evolve a mechanism to allow $CO_2$ uptake from the atmosphere, for photosynthesis, and the release of $O_2$ and water vapour. Water vapour release, also termed evapotranspiration (Young et al., 2006), cools leaves and powers transpiration-driven nutrient uptake from the soil which is paramount for plant growth and biomass production. To allow gas and water exchange between the atmosphere and the internal plant tissues, plants evolved pores with adjustable apertures, termed stomata (Beerling and Franks, 2009).

Stomata are microscopic pores (fig. 1.1) found in the epidermal cell layer of all aerial plant parts of most vascular plants and land plants (Beerling and Franks, 2009). Each pore is flanked by two specialised epidermal cells, the stomatal guard cells (GCs) which regulate the aperture of the pore. By increasing or decreasing stomatal aperture, precise regulation of $CO_2$ uptake, photosynthetic rate, transpiration and water loss can be maintained in the short term. In the longer term, plant water use and photosynthetic capacity can be controlled by alterations in stomatal development. Both these adjustments occur in response to environmental signals.

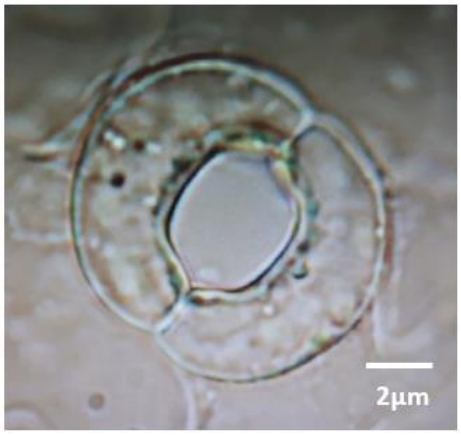


**Figure 1.1 Stomatal pore of *Arabidopsis thaliana*.** Image obtained using an Olympus BX50 microscope using Motic Images software and adjusted in ImageJ.

### **1.1.1** Stomatal movement

Stomatal aperture is regulated by the circadian clock, to ensure water preservation during the night when photosynthesis does not occur and there is no requirement for stomata to remain open for $CO_2$ uptake (Hassidim et al., 2017). However, plants can rapidly adjust stomatal aperture in response to many environmental signals, such as light and water availability, as well as endogenous hormones (Kollist et al., 2014).

By exploiting ion fluxes, mainly the movement of $K^+$ ions, and water movements, to alter GC hydrostatic pressure, rapid changes in stomatal aperture can occur (Thiel and Wolf, 1997). A host of ion-specific channels, pumps and transporters in the GC plasma membrane (PM) allow ions to move across the PM in a controlled fashion, altering the intracellular concentration of osmolytes. Resulting osmotic forces generated by differing concentrations of ions intra- versus extracellularly either draw water into the GC, or force water out. (Kollist et al., 2014). Influx and retention of water in the GCs cause an increase in cell turgor which expands the GCs, forcing them to bend away from one another, widening the space between them and opening the stomatal pore. Conversely, osmolyte and water efflux lead to a reduction in GC turgor, making the GCs flaccid, resulting in a reduction in pore aperture.

One of the most well-studied stomatal opening mechanisms is that in response to light. Both blue and red light can induce stomatal opening (Lurie, 1978), with blue light acting as a dominant signal (Chen et al., 2012). Light-induced stomatal opening will be further discussed in section 1.2. Stomatal closure has mostly been studied in the context of drought and an increase in the phytohormone abscisic acid (ABA). When a plant experiences low relative humidity or soil drought, ABA is synthesised *de novo* in the GCs (Bauer et al., 2013) and/or release from conjugates, mainly ABA glucosyl ester, by beta-glucosidase activity (Dietz et al., 2000). ABA perception by GCs ultimately leads to altered activity of the PM $H^+$ ATPase (Goh et al., 1996) and of vacuolar and PM ion channels (Blatt et al., 1990; Lemtiri-Chlieh and MacRobbie, 1994). $K^+$ ions are shuttled out of the GCs, causing water loss, a decrease in turgor, and stomatal closure (Daszkowska and Szarajeko, 2013).

Adjustment of stomatal aperture enables plants to rapidly respond to the prevailing environmental conditions. Such adjustments are, however, energetically taxing (McLachlan et al., 2016). Plants have therefore evolved the ability to make longer-term adjustments to stomatal conductance by altering the number and/or density of stomata on their leaves.

### **1.1.2** Stomatal development

Stomata are more abundant on the abaxial side of Arabidopsis leaves, and are separated from each other by at least on pavement cell following the one-cell spacing rule (Geisler et al., 2000). Critical factors involved in stomatal patterning include the subtilisin-related protease STOMATAL DENSITY AND DISTRIBUTION1 (SDD1; Von Groll et al., 2002); the Leucine-rich repeat-containing receptor-like protein TOO MANY MOUTHS (TMM; Nadeau and Sack, 2002); the mitogen-activated protein kinase kinase kinase YODA (YDA; Bergmann et al., 2004); and members of the ERECTA family (Shpak et al., 2004). SDD1 negatively regulates the number of stomatal precursor cells which differentiate to initiate the guard cell lineage (Berger and Altmann, 2000), while a mutation in *TMM* or *ERECTA* causes stomatal clustering (Yang and Sack, 1995), providing evidence for their involvement in stomatal spacing. Bergmann et al. (2004) found that meristemoid divisions were altered in *yda* seedlings compared with WT; most meristemoid cells went on to form guard cells, whereas in WT they form guard or pavement cells. Allowing at least one epidermal pavement cell between each pore is thought to allow efficient gas exchange (Papanatsiou et al., 2016).

GCs are specialised epidermal cells that have undergone several transitions to reach the fully differentiated stage which allow them to act as regulators of stomatal pore aperture. They are not fully committed until they have transitioned to a mature GC - development can be arrested at any stage to later be resumed, or the cell may even exit the lineage completely and never become a fully differentiated GC (Zoulias et al., 2018). This allows the plant to precisely adjust the number of stomata in response to environmental conditions and other internal signals (Han and Torii, 2016; Zoulias et al., 2018). In addition, mature leaves relay information about $CO_2$ and light signals from the environment to developing leaves and thereby triggering appropriate developmental procedures (Lake et al., 2002; Casson and Hetherington, 2014).

Figure 1.1.2 depicts the cell divisions and transitions along the stomatal lineage. It begins with a subset of protodermal cells which turn into meristemoid mother cells (MMC). These then divide to form one meristemoid cell and one stomatal lineage ground cell (Dong et al., 2009). This step is driven by the basic helix-loop-helix (bHLH) transcription factor SPEECHLESS (SPCH; Pillitteri et al., 2007), with the aid of SCREAM/2 (SCRM/2; Kanaoka et al., 2008). The next step - the transition of the meristemoid into a guard mother cell (GMC) is coordinated by MUTE by triggering expression of genes involved in the cell cycle which forces the transition (Han et al., 2018), also with the help of SCRM/2 (Pillitteri et al., 2007; Kanaoka et al., 2008). The meristemoid can regenerate itself and also produce further SLGCs, expanding the total number of cells in the epidermis. The final transition in the lineage, GMC transition into GC, is promoted by the transcription factors FAMA (Ohashi-Ito and Bergmann, 2006) and SCRM/2 which inhibit proteins driving the cell cycle. As the cell cycle is arrested, the GMC can become fully differentiated GCs. SPCH, MUTE and FAMA are all essential for stomatal development, as experiments have shown that mutations in either of these factors causes development of abnormal stomata (Zoulias et al., 2018).

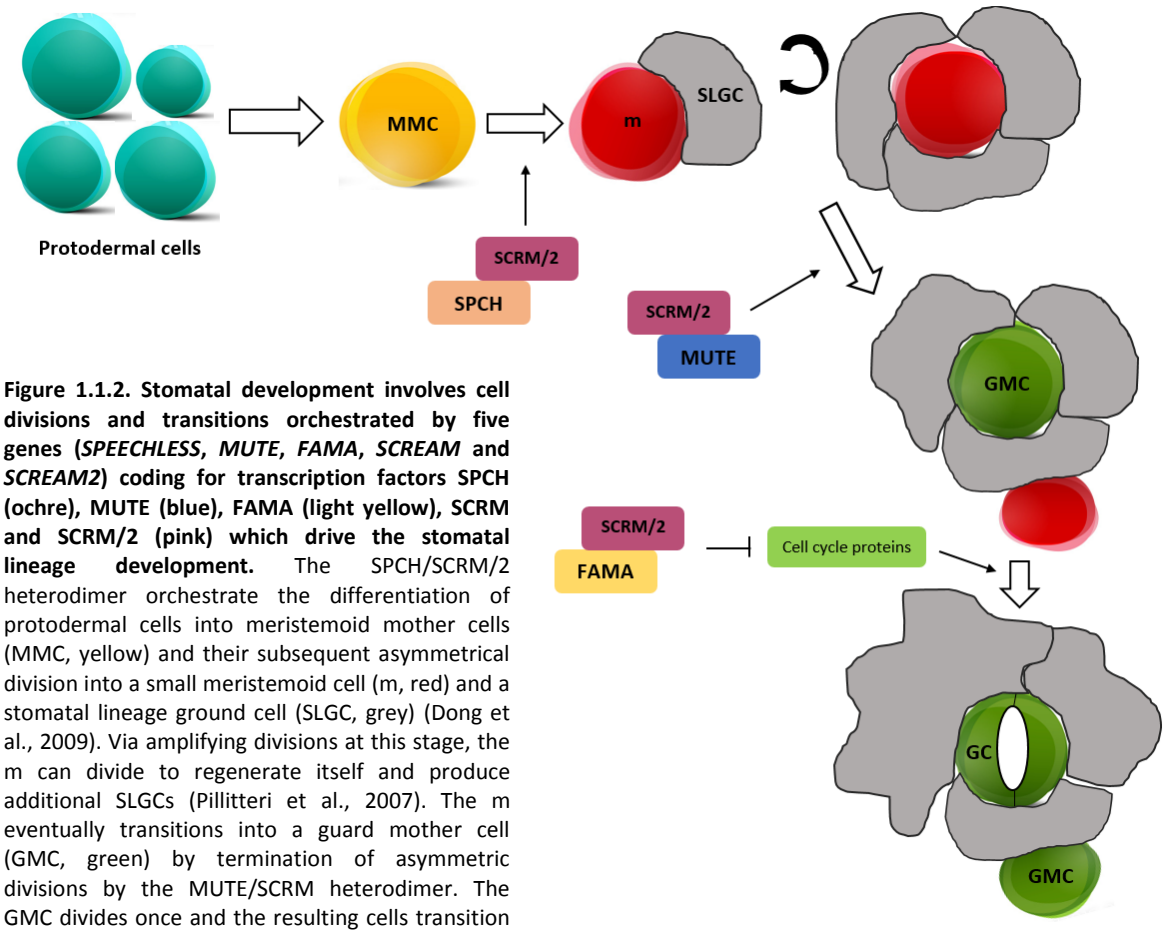


**Figure 1.1.2. Stomatal development involves cell divisions and transitions orchestrated by five genes (*SPEECHLESS*, *MUTE*, *FAMA*, *SCREAM* and *SCREAM2*) coding for transcription factors SPCH (ochre), MUTE (blue), FAMA (light yellow), SCRM and SCRM/2 (pink) which drive the stomatal lineage development.** The SPCH/SCRM/2 heterodimer orchestrate the differentiation of protodermal cells into meristemoid mother cells (MMC, yellow) and their subsequent asymmetrical division into a small meristemoid cell (m, red) and a stomatal lineage ground cell (SLGC, grey) (Dong et al., 2009). Via amplifying divisions at this stage, the m can divide to regenerate itself and produce additional SLGCs (Pillitteri et al., 2007). The m eventually transitions into a guard mother cell (GMC, green) by termination of asymmetric divisions by the MUTE/SCRM heterodimer. The GMC divides once and the resulting cells transition into guard cells (GCs) with the aid of inhibition of proteins that drive cell cycle transition by the FAMA/SCRM/2 heterodimer (Zoulias et al., 2018). Figure adapted from Han and Torii (2016).

## 1.2 Light

Plants are photoautotrophs, utilising photons for photosynthetic activity to provide energy for growth and development. Light also acts as a signal to inform plants about their surroundings, controlling a suite of developmental responses, termed photomorphogenesis. Light can provide plants with information about neighbour proximity, time of day or year and pending light-stress, requiring protective measures. Plants perceive light via specialised photoreceptors. Light of different qualities (wavelength) and quantities (fluence rates) are perceived by the photoreceptors which launch signalling cascades resulting in wavelength- and/or fluence rate-specific responses.

Different wavelengths of light have different effects on stomatal aperture. During the daytime, abundant blue (approx.450-490nm) and red (approx. 620-78nm) light in sunlight mediate stomatal opening in a phototropin (phot)- (Kinoshita et al., 2001), cryptochrome (cry)-(Mao et al., 2005), and phytochrome (phy)-(Wang et al., 2010) dependent manner. Red light can additionally induce stomatal opening via photosynthesis in mesophyll and guard cells (Mott et al., 2008; Suetsugu et al., 2014), enhancing blue light-induced stomatal opening (Shimazaki et al., 2007). Conversely, far-red light was shown to reverse low fluence red and green light-induced stomatal opening in Orchid (Talbott et al., 2002). This wavelength responsivity may have evolved to coincide with the

absorbance of chlorophyll, ensuring that stomata are kept open for $CO_2$ uptake (Assmann and Shimazaki, 1999).

The mechanism by which GCs open stomata in response to blue light involves the phot photoreceptors phot1 and phot2. Phot1/2 are protein kinases that auto-phosphorylate two Serine residues in the phot activation loop (Inoue et al., 2008). The phosphorylated (and thereby activated) phot, in turn, phosphorylates the protein kinase BLUE LIGHT SIGNALING1 (BLUS1; specific to GCs), which, via a type 1 protein phosphatase (PP1; Takemiya et al., 2006) and with the help of 14-3-3 proteins (Tseng et al., 2012), ultimately activates the PM $H^+$ ATPase (Kinoshita and Shimazaki, 1999). $H^+$ extrusion from the GC cytoplasm leads to hyperpolarisation of the PM (Shimazaki et al., 2007). Voltage-gated $K^+$-channels are activated via this hyperpolarisation and $K^+$ ions can enter the GC cytoplasm. $K^+$ uptake is balanced by the uptake of $Cl^-$ and malate (Inoue and Kinoshita, 2017). Influx of $K^+$ ions drive an increase in water uptake into the GCs via osmotic forces (Inoue and Kinoshita, 2017) which in turn increases GC turgor, forcing the GCs to bend away from each other, and opening the stomatal pore. Stomatal opening is energetically taxing and ATP for this process is obtained from starch and triacylglycerol breakdown (Horrer et al., 2016; McLachlan et al., 2016).

In addition to the effect of light on stomatal aperture, light quantity can also control stomatal patterning (Casson and Gray, 2008). An increase in light intensity has been shown to increase both stomatal density (the number of stomata per unit area) and stomatal index (the ratio of the number of stomata in a unit area divided by the total number of epidermal cells plus stomata in that area (Lake et al., 2002). The photoreceptor, phyB, and the bHLH transcription factor, PHYTOCHROME INTERACTING FACTOR 4 (PIF4) have been shown to be involved in stomatal development in response to light quantity (Casson et al., 2009).

### **1.2.1** UV-B

Ultraviolet radiation is an intrinsic component of sunlight. Owing to the short wavelength of UV photons, UV radiation has high energy. This high energy radiation can be very harmful to biological molecules which readily absorb it by altering their fundamental structure (Gill et al., 2015). The UV spectrum is divided into three wavelength intervals (UV-A, UV-B, UV-C) where ultraviolet C (UV-C) comprises the shortest (and highest energy) wavelength at 100-290nm. Virtually all UV-C radiation is absorbed by the Earth's atmosphere, so while UV-C is very damaging to biological molecules, very little of it reaches the biosphere. Ultraviolet A (UV-A; 320-400nm) is the least harmful and is visible to certain species of birds and insects (Stanford Solar Center, 2015). UV-A is absorbed by blue light photoreceptors (Christie et al., 2015) and has been shown to have certain ameliorating properties on UV-B damage in plants (Krizek, 2004). Most UV-B (290-320nm) radiation is absorbed by ozone in the stratosphere (Frohnmeyer and Staiger, 2003). The radiation that does reach the biosphere is, however, very biologically active. UV-B causes stress on biological systems by altering the structure of DNA and damaging membranes and proteins (Schwarz, 1998). For example, UV-B can induce cyclobutene pyrimidine dimers (CPDs) which negatively affect the ability of RNA/DNA polymerase to read the genetic code, resulting in defective transcription and DNA replication, respectively (Frohnmeyer and Staiger, 2003).

Plants in natural environments will experience daily exposure to UV-B radiation. The fluence-rates of UV-B reaching the biosphere are dependent on ozone layer coverage, atmospheric particle constituents, both natural (eg. oxygen molecules) and pollutants (eg. aerosols in the troposphere) which contribute to UV-B absorption (IARC, 2012), cloud cover, surface reflectance, buildings, or natural structures (Hartmann et al., 2013), and shading by other plants. UV-B exposure must therefore be considered dynamic across and at the Earth's surface (Watanabe et al., 2011).

The stratospheric ozone layer protects biomolecules from harmful UV-B radiation (Staehelin et al., 2001). A depletion of the ozone layer was, however, identified in the 1980s. The realisation that anthropogenic factors (eg. aerosol pollution) were contributing to this ozone depletion resulted in the creation of the Vienna convention for the protection of the ozone layer in 1985 (EPA, 2017). NASA reported in January 2018 that the ozone layer is now recovering following a reduction in anthropogenic emissions of chlorofluorocarbons (CFCs; Strahan and Douglass, 2018). Despite this measurable recovery it will take decades for the ozone hole to fully recover, if ever it can completely. Anthropogenic emissions and production/invention of other chemicals continue, and it is not clear what effect these all have on the atmospheric composition, which could result in a continued depletion of ozone and increased UV-B exposure.

Regardless of the status of the ozone layer, the effect of UV-B radiation on plants is an important, yet poorly studied subject. Apart from acting as a stress signal and being potentially harmful to plants, lower dose UV-B also acts as a photomorphogenic signal which can regulate plant development.

### **1.2.2** Plant responses to UV-B

UV-B radiation triggers photomorphogenic responses such as inhibition of hypocotyl elongation in etiolated tomato seedlings (Ballare et al., 1995), sunflower (Ros and Tevini, 1995), and Arabidopsis (Hayes et al., 2014) and prompts cotyledon expansion (Boccalandro et al., 2001). UV-B was also observed to inhibit shade avoidance in Arabidopsis (Hayes et al., 2014) and coriander (Fraser et al., 2017). Shade avoidance responses occur in dense canopies in response to low levels of blue light and reductions in red-far red ratio (low R:FR). These include elongation of hypocotyls and petioles, leaf hyponasty and reduced leaf surface area, and enable plants to outcompete neighbouring plants for sunlight (Fiorucci and Fankhauser, 2017). An increased R:FR ratio as the plant outgrows its neighbours and is exposed to direct sunlight results in halted hypocotyl/petiole elongation. Similarly, UV-B seems to provide the plant with a signal of direct sunlight inhibiting hypocotyl and petiole elongation (Ros and Tevini, 1995; Hectors et al., 2007; Hayes et al., 2014).

It has been hypothesised that the broad range of responses of plants to UV-B are a means to protect against the UV-B radiation itself. It is achieved by producing sunscreens in the form of protective pigments and antioxidants (Caldwell et al., 1983; Strid and Porra, 1992; Christie and Jenkins, 1996; Kalbin et al., 1997), enhancing DNA repair, and adjusting plant architecture to reduce UV-B exposure (Tilbrook et al., 2013). These changes occur via changes in gene expression, for example upregulation of antioxidant biosynthesis pathway component genes (Jenkins et al., 2001). This increase in levels of antioxidants and flavonoids has been shown to correlate with an increase in intracellular reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$) and NO, post UV-B exposure (Zhang et al., 2009). UV-B has also been observed to regulate stomatal movement and stomatal development (these will be further discussed in chapter 3 and 4, respectively) and to inhibit thermomorphogenesis (plant growth in response to elevated temperature) in Arabidopsis seedlings (Hayes et al., 2017).

### **1.2.3** UV-B signalling

The UV-B signalling pathway is initiated as UV-B is perceived by the UV-B photoreceptor, UVR8. UVR8 was first identified by Kleibenstein et al. (2002) in a screen for mutants hypersensitive to UV-B irradiation. Its physiological role in UV-B tolerance was later described by Favory et al. (2009), and its role as the UV-B photoreceptor by Rizzini et al., (2011). In absence of UV-B, UVR8 exists in a homodimeric form, held together by salt-bridges, inter-molecular hydrogen bonds, and cation TT-

interactions, principally between tryptophan (Trp) and arginine (Arg) residues at the homodimeric interface (Christie et al., 2012). UVR8 is enriched with Trp residues, which also naturally absorb UV-B light. Trp-285 and Trp-233 of the UVR8 protein serve as the UV-B-absorbing chromophore (Wu et al., 2012) with minor contribution from Trp-337 which is not essential in UV-B perception (O'Hara and Jenkins, 2012). In contrast to other photoreceptor proteins which commonly possess co-factors as the light-absorbing chromophore, the capability of intrinsic Trp residues to absorb UV-B allow the UVR8 protein to act as a photoreceptor (Christie et al., 2012). Upon UV-B exposure, Trp-285 and Trp-233 excitation results in disrupted intermolecular bonds, specifically the cation TT interactions with Arg-286 and Arg-338 are broken. This de-stabilises the H-bonds at the dimer interface, and the homodimeric structure is disrupted (Christie et al., 2012).

UVR8 monomerisation exposes a binding site for CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) which was initially identified via a loss of function mutant displaying constitutive photomorphogenesis (Deng et al., 1991). COP1 is an E3 ubiquitin ligase (Seo et al., 2003) targeting molecules for degradation by the 26S proteasome via ubiquitination. It was shown by Ang et al. (1998) that COP1 negatively regulates ELONGATED HYPOCOTYL 5 (HY5); Osterlund et al. (2000) showed that the level of HY5 coincide with photomorphogenic responses. HY5 is a transcription factor involved in the light response pathway, for example, as a positive regulator of hypocotyl elongation (Oyama et al., 1997). In absence of UV-B, COP1 targets HY5 for degradation, however upon UV-B exposure and UVR8 monomerisation and binding of UVR8 to COP1 (Yin et al., 2015), HY5 is allowed to accumulate in the nucleus and *HY5* expression is activated (Oravecz et al., 2006). HY5 signalling leads to inhibition of hypocotyl elongation - a characteristic UV-B response (Ballare et al., 1995; Gruber 2010). UVR8 dimers can regenerate from the monomer pool and do so with the help of REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2 (RUP1 and RUP2) (Gruber et al., 2010). Initiation of *RUP1/2* expression is dependent on UVR8, COP1 and HY5 activity in a UV-B-induced manner, creating a negative feedback loop in UV-B signalling.

Most UVR8 molecules are cytoplasmic, both in absence and presence of UV-B, however, upon UV-B exposure, UVR8 accumulates in the nucleus (Kaiserli and Jenkins, 2007). The majority of UV-B responses involve direct gene expression alterations, such as the upregulation of pigment biosynthesis genes (Christie and Jenkins, 1996), but also affect longer term cell signalling, for example auxin biosynthesis (Hayes et al., 2014). Despite nuclear accumulation, the majority of UVR8 molecules remain cytoplasmic. No major roles for UVR8 in the cytoplasm have been reported thus far, but cannot be dismissed (Tilbrook et al., 2013).

## 1.3 Elevated temperature

Natural sunlight provides heat. Temperature varies greatly, both diurnally and seasonally, across the globe. Temperature is influenced by latitude, longitude and altitude, but also by smaller differences in environmental surroundings, such as the degree of shade (Armson et al., 2012). Plants have evolved mechanisms to withstand fluctuations in temperature, such as the production of heat-shock proteins (Hsp; Wang et al., 2004), anti-freeze proteins (AFPs; Griffith and Yaish, 2004; Knight and Knight, 2012), alterations in leaf orientation to reduce/increase direct radiation (van Zanten et al., 2010) and alterations in architecture to facilitate leaf cooling (Crawford et al., 2012). Temperature tolerance vary with plant species. In Arabidopsis, it has been suggested that 22-27$^{o}$C is a moderate temperature elevation, 27-30$^{o}$C a high temperature, and 37-42$^{o}$C an extremely high temperature (Liu et al., 2015). Arabidopsis is native to Eurasia and Africa, and also grows in North America (TAIR, 2018). Native conditions may vary, particularly with regards to temperature, from those applied in the lab. For example, in this study, Arabidopsis was grown at 22$^{o}$C (day, control) or 28$^{o}$C (day,

experimental treatment temperature) and 20°C (night, control) or 22°C (night, experimental treatment temperature); whereas the monthly mean temperature of a summer in the UK reaches approximately 15-16°C in July/August (National Statistics, 2018). Furthermore, basic physiological activities may differ depending on where the accession originated. Adams et al. (2016) noted that, for example, baseline photosynthetic capacity varied between Arabidopsis accessions native to Sweden, Poland and Italy, which may reflect the conditions in which the accessions evolved.

According to the Intergovernmental panel on climate change (2014), earth surface warming has shown an upwards trend since 1900 and is projected to continue to rise for the foreseeable future (table 1.3; fig. 1.3). This is a problem for plants, as they will find themselves in hotter conditions than they have evolved to endure, and their ability to adapt to these conditions may not be rapid enough. There is a need for further understanding of how elevated temperatures impact plant productivity and survival.

| Case | Temperature Change (°C at 2090-2099 relative to 1980-1999)[a] | | Sea Level Rise) (m at 2090-2099 relative to 1980-1999) |
|---|---|---|---|
| | Best estimate | *Likely* range | Model-based range excluding future rapid dynamical changes in ice flow |
| Constant Year 2000 concentrations[b] | 0.6 | 0.3 – 0.9 | NA |
| B1 scenario | 1.8 | 1.1 – 2.9 | 0.18 – 0.38 |
| A1T scenario | 2.4 | 1.4 – 3.8 | 0.20 – 0.45 |
| B2 scenario | 2.4 | 1.4 – 3.8 | 0.20 – 0.43 |
| A1B scenario | 2.8 | 1.7 – 4.4 | 0.21 – 0.48 |
| A2 scenario | 3.4 | 2.0 – 5.4 | 0.23 – 0.51 |
| A1FI scenario | 4.0 | 2.4 – 6.4 | 0.26 – 0.59 |

**Table 1.3. Predicted global surface warming and sea level rise according to different scenarios.** The intergovernmental panel on climate change (2007) assessed data from climate models, Earth System Models, and Atmosphere-Ocean General Circulation Models to produce the above table of projected global temperature changes. Scenarios B1-A1F1 are based on six possible future global anthropogenic emission levels of greenhouse gases and predictions made based on these emission levels (Image from IPCC, 2007).

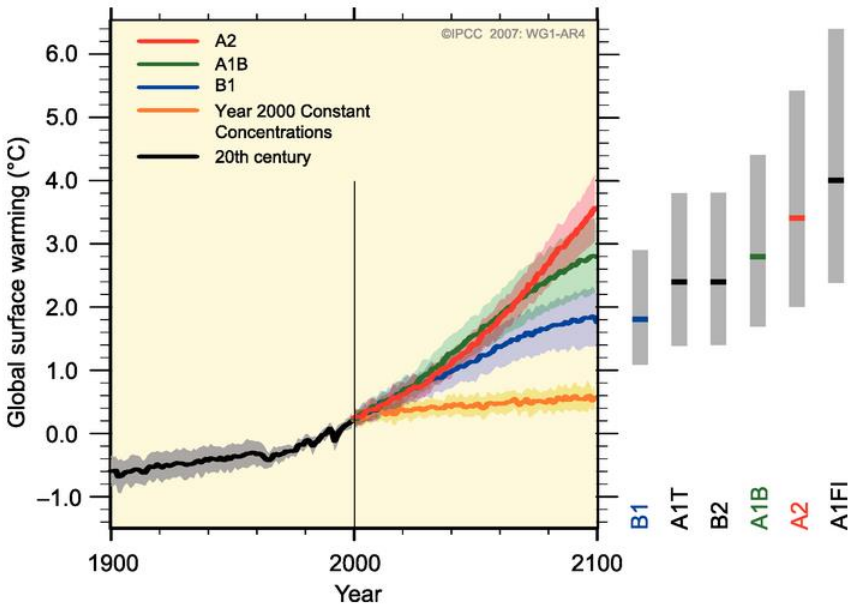

**Figure 1.3 Visual representation of projected scenarios mentioned in table 1**. The Intergovernmental Panel on Climate Change analysed data beginning 1900 until 2007; using various climate models predicted global surface warming according to six different scenarios based on six different emission levels. Even if the emission levels of year 2000 remain constant, global temperatures are predicted to rise by 0.3-0.9$^{o}$C in 2000-2100 century. (Image from IPCC, 2007).

### **1.3.1** Physiological effects of high temperature

Thermomorphogenesis is defined as morphological changes in response to changes in temperature, below stress levels of heat (Quint et al., 2016). These changes include hypocotyl and petiole elongation, leaf hyponasty, and the production of smaller and thinner leaves. High temperature (28$^{o}$C) has been shown to reduce stomatal density in Arabidopsis (Crawford et al., 2012), but cause an increase in soybean (Jumrani et al., 2017). Elevated temperatures (>25$^{o}$C) also mediates stomatal opening in leaves of bean (Feller, 2006) mediated by an influx of $K^{+}$ (Rogers et al., 1979). The effects of elevated temperature and water stress was investigated in four plant species native to Israel by Schultze et al. (1973) who found that while stomatal aperture increased with increasing temperatures, water stress antagonised the response, closing stomata. Adaptations and responses to elevated temperatures are likely in place to enhance cooling as higher transpiration rates and cooler leaves have been observed in combination with morphological changes (Bridge et al., 2013; Crawford et al., 2012).

### **1.3.2** Temperature perception and signalling

Precise mechanisms of temperature perception in Arabidopsis remain elusive. Considering the sensitivity of biological molecules to temperature (structure and integrity), many of these could be considered temperature "receptors" (Franklin et al., 2014). However, some photoreceptors have been established as thermoreceptors with specific, temperature-dependent responses in Arabidopsis. Indeed, phot1/2 and phyB have been shown to function as both light- and temperature-receptors (Fujii et al., 2017; Legris et al., 2016; Jung et al., 2016). It is however, currently unclear how widespread their action is in regulating global temperature responses within cells.

A central player in ambient temperature signalling is the bHLH transcription factor PIF4. *PIF4* abundance is increased in plants grown in or transferred to elevated temperatures (Koini et al., 2009). PIF4 mediates high temperature responses such as hypocotyl elongation by upregulating auxin biosynthesis genes such as *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and *YUC8* (Franklin et al., 2011; Sun et al., 2012 figure 1.3.2c). Auxin binds to the auxin-receptor protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Dharmasiri et al., 2005) which in turn upregulates the expression of auxin-responsive genes SMALL AUXIN UP RNA (SAUR) which regulate elongation growth (Spartz et al., 2012). Several signalling pathways converge on PIF4 to influence responses to ambient conditions, and PIF4 abundance and action appears to be tightly regulated spatially and temporally to allow fine-tuned plant growth (Quint et al., 2016). *PIF4* expression is regulated by the circadian clock and the evening complex (EC: EARLY FLOWERING 3 (ELF3) and 4 (ELF4) and LUX ARRYTHMO (LUX)). Nieto et al. (2015) also showed that PIF4 activity could be regulated at high temperature by direct ELF3:PIF4 interaction.

Phytohormones can also influence PIF4 action. Gibberellic acid (GA) and brassinosteroids (BR) regulate PIF4 action via the "BAP module", which includes transcriptional repressor BRASSINOSTEROID SIGNALLING POSITIVE REGULATOR (BZR1) and AUXIN REGULATING FACTOR 6 (ARF6), and PIF4 (Oh et al., 2014). GA degrades DELLA proteins which normally bind to and inhibit

PIF4 action in light signalling (de Lucas et al., 2008). This mechanism is also required for thermomorphogenesis but not sufficient to induce the thermomorphogenic responses (Stavang et al., 2009). Furthermore, chromatin accessibility and methylation patterns, as well as some known, and probably several unknown, kinases/phosphatases all influence PIF activity and action (fig. 1.3.2). Despite the central role of PIF4 in temperature signalling, it was shown to lack a role in high temperature-mediated stomatal opening (Kostaki et al., manuscript in preparation), but has been shown to prevent expression of SPCH which initiate the stomatal lineage in Arabidopsis (Lau et al., 2018).

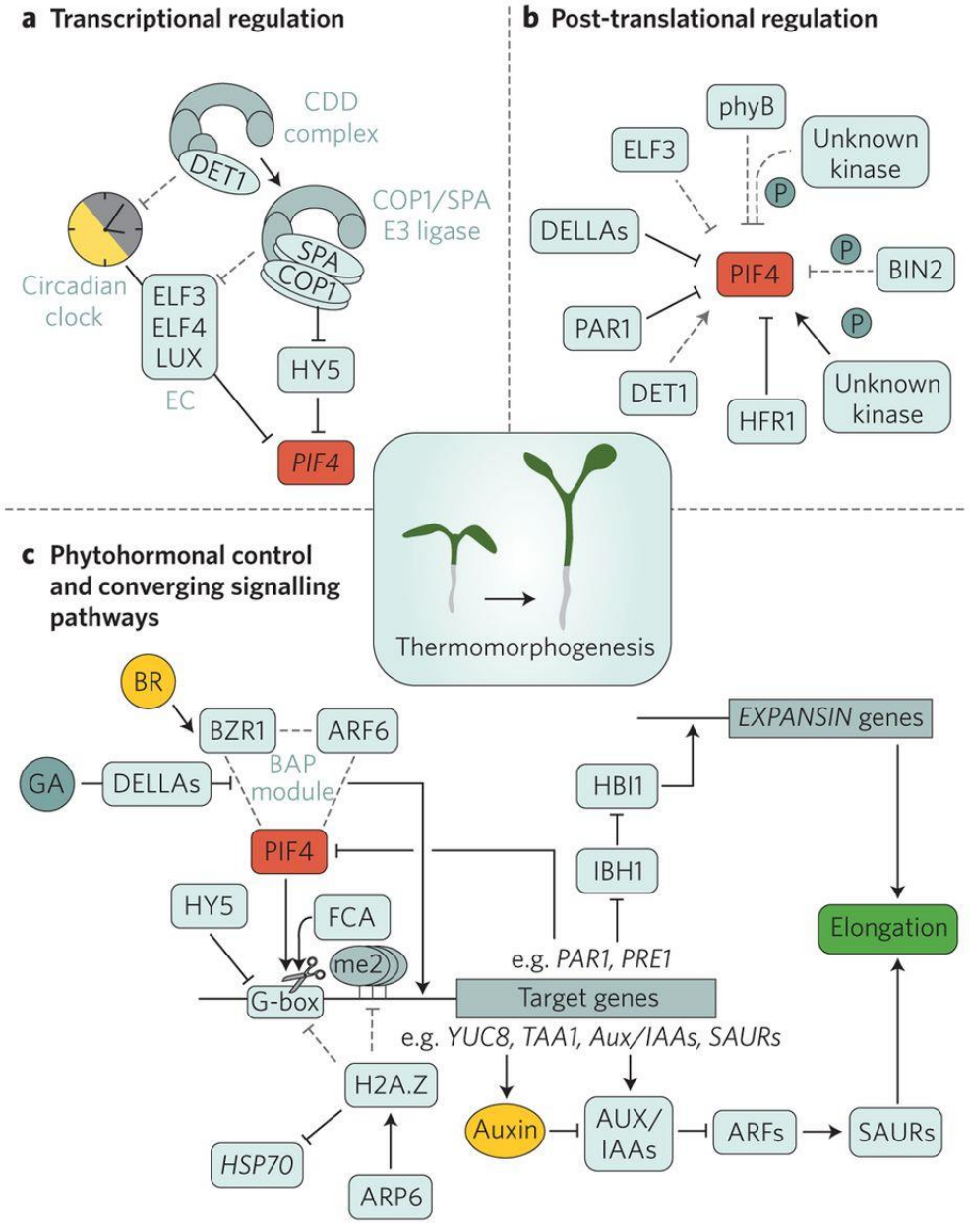


**Figure 1.3.2**. **Multiple signals influence PIF4 abundance and activity.** a) Regulation of *PIF4* transcript abundance occurs via the circadian clock and the evening complex, as well as via COP1-SPA and HY5 action. b) PIF4 is post-translationally regulated by several known and unknown components, for example DELLA proteins which inhibit PIF4 action in light signalling. c) Brassinosteroid and Gibberellins regulate PIF4 action via the "BAP

module" involving for example transcriptional repressor brassinosteroid signalling positive regulator (BZR1). Auxin biosynthesis is one target for PIF4. (Image: Quint et al., 2016).

## **1.4** UV-B and high temperature signalling crosstalk

UV-B is a component of natural sunlight, and sunlight provides heat. This means that plants exposed to strong direct sunlight generally experience UV-B irradiation and high temperatures simultaneously. Exceptions include alpine conditions where high UV-B irradiation (due to high altitude) is combined with very low ambient temperatures, and rainforest biomes where elevated temperatures and a humid climate are combined with low UV-B irradiation below the canopy. However, most areas where important crops plants are grown are in direct sunlight at varying temperatures. With an increase in global temperatures in these conditions and an uncertain status of the stratospheric ozone layer, understanding plant responses in such conditions can be vital for work in mitigating any negative effects on crop production.

As previously discussed, UV-B has been shown inhibit thermomorphogenesis in Arabidopsis (Hayes et al., 2017), reducing hypocotyl and petiole growth. Other effects of UV-B on high temperature-induced responses are largely unexplored and to date our knowledge on the effects of UV-B on stomatal behaviour during elevated temperatures is limited.

## **1.5 Aims and objectives**

The aims of this work were to elucidate the combined effects of elevated temperature and UV-B irradiation on stomatal aperture and stomatal development as well as probe the temperature and UV-B signalling pathways that lead to this response for components involved. This thesis focuses on the responses of stomata of *Arabidopsis thaliana* to elevated temperature and UV-B radiation in a laboratory setting. The questions were addressed through the following:

- Measure stomatal apertures after different temperature and UV-B treatments in epidermal peels of *Arabidopsis thaliana* accessions

- Investigate the viability of cells after the application of the experimental conditions to ensure results were not due to cell death

- Measure nitric oxide levels of guard cells after different temperature and UV-B treatments

- Investigate the involvement of ABA in the short-term UV-B-induced stomatal closure response

- Explore the involvement of mesophyll cells in stomatal movement by subjecting whole leaves to the temperature and UV-B treatments

- Study the effects of UV-B on stomatal development *in planta* at two different temperatures

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Seed stocks

*Arabidopsis thaliana* seeds of accessions Col-0, Wassilewskija (Ws), and *Landsberg erects* (*Ler*) were obtained from Dr Ioanna Kostaki and Dr Ashutosh Sharma (School of Life Sciences, University of Bristol, Bristol, UK). Seeds of Arabidopsis mutants *uvr8-6* and *nced3/5* have been previously described (Favory et al., 2009; Frey et al., 2011). Seeds were sterilised according to Springer protocol (Podar, 2013) using either 70% ethanol (EtOH) or chlorine gas. For the EtOH method, seeds were washed in 70% EtOH for 10 min and then washed twice with sterilised de-ionised water before immediately planted onto agar (described below). Where chlorine gas method was used for sterilising seeds, seeds were put into open Eppendorf tubes in a sealable plastic box. Inside the plastic box was a 200 mL beaker containing 100 mL 100% bleach (5% available as chlorine) and 3mL hydrochloric acid. The box was sealed and left for 3-3.5 h. Seeds sterilised using this method were kept up to 1 week.

## 2.2 Stomatal aperture

### **2.2.1** Growth conditions

Arabidopsis seeds (Col-0, Ws, Ler, *nced3/5*, *uvr8-6*) were planted on ½ strength Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, US; Murashige and Skoog, 1962) containing 1% w/v sucrose, 0.6% w/v agar, pH 5.8, according to Springer protocol (Podar, 2013). Seeds were stratified in darkness at +5$^{o}$C for 48 h before being transferred to a Microclima growth cabinet (Snijders Scientific, Tilburg, The Netherlands). Temperature was maintained at 22$^{o}$C and relative humidity (RH) at 70% in a 10 h photoperiod (10 h light: 14 h dark). White light was provided by cool-white SYLVANIA F36W/2084-T8 BriteGro or SYLVANIA LUXLINE PLUS FHO54W/T5/840 light tubes at 100 µmol $m^{-2}$ $s^{-1}$ (400-700nm). White light was measured using a using an Ocean Optics Flame Spectrometer with OceanView 1.6.7 software. Week old seedlings were transplanted onto 3:1 ratio of compost (Sinclair all-purpose growth medium, William Sinclair Horticultural Ltd, Lincoln) and sand (Horticultural Silver Sand, Melcourt Garden and Landscape, UK). Plants were watered with de-ionised water (Purite water system, SUEZ water, Thame, UK) applied directly in to growth trays three times weekly.

### **2.2.2** Preparation of leaf epidermal peels and whole leaves

Epidermal strips from Arabidopsis (Col-0, Ws, Ler, *nced3/5* and *uvr8-6*) leaves (approx. 5x5 mm, 3 per experimental treatment from separate plants of similar development stage) were prepared from 4-6 week old seedlings using one mature, fully expanded leaf per plant. Strips were suspended in 10/0 MES/KCl buffer (10 mM MES, 0mM KCl, pH 6.15) in 50 mm petri dishes (FISHER Scientific, Loughborough, UK) briefly before being transferred onto 10mL 10/50 MES/KCl buffer (10 mM MES, 50 mM KCl, pH 6.15) at the correct experimental temperature (22$^{o}$C or 35$^{o}$C) and subjected to pre-incubation conditions (22$^{o}$C or 35$^{o}$C, no UV-B).

Two whole leaves from different plants were used for whole-leaf bioassays. Leaves were floated abaxial side-up on the buffer to match epidermal peel procedure. Leaves were floated on 10 mL KCl buffer for 2 h at 22$^{o}$C or 35$^{o}$C in absence of UV-B, before transferred to fresh buffer and returned to the same temperatures ± UV-B. Leaves were peeled immediately post-exposure to produce epidermal strips for microscopy examination. Simultaneously, one epidermal peel per condition,

from the same batch of seedlings, was used as a control for the procedure. 10/0 holding buffer was heated to ~35°C for peeling the leaves that were exposed to this temperature, to reduce the risk of any closure effects that may be induced by the use of room temperature (22°C) holding buffer while peeling (approx. 3 min per leaf).

### **2.2.3** Bioassay conditions

Two glass fish tanks were filled with approximately 25L water each and heated using Julabo ED (v.2) GB 9A/8A heat generators (Julabo Labortechnic, Seelbach, Germany). Temperatures were maintained by the generators, and a tap water cooling system, throughout the experimental time periods. Trays with slots for 50mm petri plates were suspended on the water surface, allowing constant petri plate-water contact. This ensured that buffer temperature was maintained throughout the experiment.

Samples were pre-incubated for 2 h at 22°C or 35°C, in the absence of UV-B, to acclimatise to the temperatures and induce stomatal opening, before being subjected to experimental UV-B conditions. Temperature conditions were decided based on maximum stomatal opening response, explored in previous experiments carried out at the School of Life Sciences, University of Bristol (Kostaki et al., manuscript in preparation), and the temperature range within which Arabidopsis seedlings are generally sub-stress level (Liu et al., 2015).

Samples were transferred after 2 h pre-incubation onto fresh 10/50 MS/KCl buffer. Half the samples were exposed to 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B (280-315nm), supplied by PHILIPS Narrowband TL 20W/01 - RS Ultraviolet-B light bulbs, for 3 h. The other half received no UV-B exposure. White light at a fluence rate of 75-80 µmol $m^{-2}$ $s^{-1}$ was supplied from underneath the tanks by CROMPTON LAMPS 13W white A10 light bulbs. A plastic lattice grid was used to diffuse the UV-B light to achieve and maintain uniform UV-B fluence rates across all samples. A similar grid was positioned above non UV-B-treated samples to ensure comparability between experimental conditions. Light levels were measured using an Ocean Optics Flame Spectrometer with OceanView 1.6.7 software or a Skye Instruments SpectroSense 2 with a UV-B sensor.

### **2.2.4** Measurement of stomatal aperture

Epidermal peels were transferred onto microscope slides post-treatment and randomly chosen stomata situated in the middle of peels were used to measure aperture. Measurements were taken on-screen using a Leica DM IRB or an Olympus BX50 microscope with Motic Images Plus 2.0 software. Measurements of width and length were taken for all stomatal pores as width:length ratio which gives an accurate estimate of the openness of the pore. Each experiment was replicated three times on three consecutive days. All experiments commenced at 9 am, with sample examination occurring 2-3 pm (2-4 pm when two genotypes were tested). This removed any circadian effects on stomatal aperture. Three leaves per treatment were used, on which 10 stomata were measured. In total, 90 measurements per treatment were made for each aperture experiment.

### **2.2.5** Cell viability

Fluoresceine diacetate (FDA; Sigma-Aldrich) was used to measure cell viability (Widholm, 1972; Swisher and Carroll, 1980). FDA is an esterase-substrate which is readily absorbed and hydrolysed by non-specific esterases (Guilbault and Kramer, 1964) only in viable cells, producing fluorescence when excited. The hydrolysed product, fluorescein, is retained within cells and can be used to distinguish viable from non-viable cells (Sigma-Aldrich, 2005).

Epidermal peels of 4-5 week old Arabidopsis (Col-0 and *uvr8-6*) were prepared as described above, and after pre-incubation at 22°C or 35°C in the absence of UV-B for 2 h, subjected to 22°C -UV-B (least stressful treatment) or 35°C +UV-B (2.7 µmol $m^{-2}$ $s^{-1}$; most stressful treatment) for 3 h. Post-treatment peels were immediately incubated for 10 min with 10 µM FDA, then washed 3 times to remove excess probe, before mounting on standard microscope slides for examination by fluorescence microscopy. Micrograph images were obtained with a ZEISS XBO 75 (1007-981) epifluorescent microscope using Volocity Cellular Imaging software, or a Leica DM 2000 LED (Leica microsystems CMS, GmbH, Germany) and Leica Application Suite V4.4 software, excitation/emission ~490/515 nm. Micrographs were analysed, and viability calculated using ImageJ (NIH).

### **2.2.6** Nitric oxide quantification

Fluorescent probe DAF-FM diacetate (DAF-FM-DA) was used to detect NO levels of GCs according to the manufacturer's protocol. DAF-FM DA reacts with NO to produce the fluorescent product benzotriazole which is measurable and quantifiable (Kojima et al., 1998). Epidermal peels of 4-5 week old Arabidopsis (Col-0 and *uvr8-6*) were incubated for 2 h at 22°C or 35°C to induce stomatal opening and then transferred to one of four treatments: 22°C or 35°C ± 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B for 3 h, as previously described. Peels were then incubated for 30 min with 7 mM DAF-FM-DA, washed on opening buffer for an additional 30 min to allow complete de-esterification of the probes, then washed twice on fresh buffer to remove excess probe and mounted on standard microscope slides for examination by fluorescent microscopy. Micrographs were obtained using a Leica DM 2000 LED (Leica Microsystems, CMS, GmbH, Germany) fluorescent microscope (excitation/emission ~490/515 nm) and Leica Application Suite V4.4 software. Micrographs were analysed and NO quantified by pixel densitometry in ImageJ.

## **2.3 Stomatal development**

### **2.3.1** Growth conditions

Col-0 and *uvr8-6* were planted on ½ MS medium containing 1% w/v sucrose and 0.6% w/v agar, pH 5.8, and stratified in darkness at +5°C for 48 h before being transferred to a Microclima growth cabinet (Snijders Scientific, Tilburg, The Netherlands). Temperature was maintained at 22°C, RH at 70% and white light was provided by cool-white fluorescent light tubes at a photon irradiance of 100-115 µmol $m^{-2}$ $s^{-1}$ (400-700 nm). 1 week old seedlings were transplanted onto 3:1 compost-sand mix as previously described and randomly allocated to treatment groups. Treatments were 22°C ± UV-B and 28°C ±UVB. UV-B was supplied by PHILIPS TL20W/01-RS narrowband ULTRAVIOLET-B tubes and fluence rates were maintained at 2.7 µmol $m^{-2}$ $s^{-1}$.

Initially, seedlings were transplanted immediately after cotyledon expansion. This resulted in a high rate of seedling death. Seedlings subjected to 28°C were especially vulnerable, likely due to desiccation of the delicate roots. Subsequent seedlings were allowed to develop four true leaves before transplantation and allocation to experimental conditions. These leaves were marked by permanent marker and disregarded for stomatal development analysis. Seedlings subjected to 28°C required more frequent watering and an effort to prevent desiccation involved closely monitoring water status.

### **2.3.2** Sampling

Seedlings were grown until maturity (day of first bolt emergence). True leaves in the middle of the rosette were harvested and leaf casts were obtained using dental quality resin (PRESIDENT ISO 4823, Type3, low consistency polyvinylsiloxande, addition-type,; Coltene/Whaledent, Altstatten,

Switzerland) and clear nail varnish (60 second super shine nail varnish, Rimmel, London, UK). Leaf impressions were mounted on standard microscope slides using clear tape (Scotch™ transparent tape, 3M, Maplewood, USA). Three leaves selected randomly from leaf 7-12 plant were chosen for analysis. Eight plants per condition were used in total, equalling 24 measurements per condition. Images of plant morphology were obtained using a Nikon D3200 DSLR camera and processed using GIMP 2.

### **2.3.3** Measurement of stomatal density and stomatal index

Micrographs of leaf casts were obtained using a ZEISS XBO 75 (1007-981) epifluorescent microscope and Volocity Cellular Imaging software. Stomatal density was measured as number of stomata per unit area; stomatal index was calculated as number stomata divided by the number of total epidermal cells, plus stomata, in a given area. Photographs were taken for visible analysis of plant morphology.

## 2.4 Statistical analysis

Stomatal aperture, density and index, cell viability and NO level data were analysed in SigmaPlot 13.0. Data points were tested for normality and were subsequently transformed where required to achieve a normal distribution. A parametric analysis of variance (ANOVA) was performed where appropriate, as well as post-hoc Tukey-Kramer test where the ANOVA indicated significant differences between the data groups. Arcsine transformation was performed on percentage data (GC viability and stomatal index) prior to application of statistical test.

## 2.5 Method development

### **2.5.1** UV-B conditions

Narrowband UV-B light tubes were found to provide varying levels of radiation depending on the position below the tubes. This was problematic for bioassay experiments, where multiple petri plates needed to receive exactly the same dose. Many experimental setups in the literature utilise UV-B-filtering tape or rely on diacetate cellulose to reduce UV-B irradiance from the UV-B tubes. However, tape which is applied to the tubes will always produce an irregular pattern of irradiance below the tube. To obtain uniform UV-B levels in this setup, numerous materials were tested for their light-diffusing properties, as well as rig height and position. A 20 W narrowband UV-B tube was eventually used together with a white plastic lattice to obtain 2 positions below the UV-B light bulb that varied with as little as 0.05 µmol $m^{-2}s^{-1}$. The lattice allowed even light diffusion over the samples and gave a more uniform fluence rate.

### **2.5.2** Experimental set-up for whole leaf bioassays

Whole leaves of Arabidopsis were used for whole leaf bioassays. Leaves were suspended, abaxial side up, on 10/50 MES/KCl buffer for the duration of the experiment. Leaves floated on the buffer rather than becoming submerged. This was suspected to affect opening capacity due to reduced contact between the abaxial side of the leaves with the buffer. Leaves were then floated, adaxial side up, on the buffer to investigate any difference in aperture. However, this may have caused a reduction in the UV-B reaching the abaxial GCs.

# CHAPTER 3 UV-B ANTAGONISES HIGH TEMPERATURE-INDUCED STOMATAL OPENING IN *ARABIDOPSIS THALIANA*

## 3.1 Introduction

Plants manage water status and gas exchange via stomatal pores in the epidermis of leaves. Pore aperture is governed by a pair of flanking GCs. The GCs use changes in ion fluxes (mainly $K^+$) and osmotic forces to alter GC turgor, thereby controlling pore openness. UV-B has been shown to mediate complex responses in stomatal behaviour, mediating both opening and closure (Jansen and Noort, 2000). Early experiments showed a decrease in stomatal conductance in pea, *Commelina communis* and oilseed rape (Nogués et al., 1999) and in stomatal aperture in rice (Dai et al., 1995) in response to UV-B. More recent studies have shown that the main effect of higher levels of UV-B is stomatal closure. This has been tested in Arabidopsis (Tossi et al., 2009; He et al., 2013; Tossi et al., 2014). Tossi et al. (2014) showed that UVR8 mediates UV-B perception in the stomatal closure response. The same study also showed that COP1 and HY5/ELONGATED HYPOCOTYL HOMOLOGUE (HYH) regulate stomatal closure, via ROS signalling. NO was found to be essential in the stomatal closure response regardless of $H_2O_2$ levels. NO was first shown by García-Mata and Lamattina (2001) to induce stomatal closure in wheat and bean when applied externally. Wilson et al. (2009) demonstrated that NO is involved in stomatal closure in turgid leaves in response to ABA, but no such involvement was seen in leaves under severe dehydration. This suggested that NO could be involved in stomatal movement in response to environmental factors other than drought, such as light and temperature, likely via a nitrate reductase (NR) mechanism (Neill et al., 2008; Santolini et al., 2017).

High levels of sunlight, and thereby high UV-B irradiation, and dry conditions often occur simultaneously. Stomata close in an ABA-mediated manner in response to drought or reduced relative humidity (Munemasa et al., 2015). ABA was shown to accumulate in response to UV-B (Tossi et al., 2009), and it was suggested by Tossi et al. (2014) that ABA may play a role in the UV-B-induced closure response either via HY5-coordination of ABA-responsive genes, or an independent mechanism. It was further suggested that ABA could trigger ROS generation via either of these mechanisms, and thereby stomatal closure. To date, no evidence confirming this suggestion has been presented.

Strong sunlight often coincides with elevated temperatures. High temperatures generally mediate stomatal opening in well-watered conditions, which has been shown in bean (Rogers et al., 1979; Hofstra and Hesketh, 1969; Feller, 2006), poplar and loblolly pine (Urban et al., 2017) at temperatures of up to 40$^{o}$C. Although high temperature-mediated stomatal opening has been observed, little is known about the signalling pathway involved. UV-B was shown to inhibit morphological changes of the thermomorphogenic response in Arabidopsis (Hayes et al., 2017) however the effects of UV-B on high temperature-induced stomatal responses have not been explored. We hypothesised that UV-B antagonises high temperature-induced stomatal opening, similar to its inhibitory effect on thermomorphogenesis. Little is known about the UV-B-induced signalling pathway leading to stomatal closure in these conditions. This work focused on three likely components of the signalling pathway involved in stomatal movement: the UV-B photoreceptor UVR8, the phytohormone ABA, and the reactive oxygen species NO.

## 3.2 Results

### **3.2.1** UV-B reduces stomatal aperture in both 22°C and 35°C in *Arabidopsis thaliana*

Epidermal peels of multiple Arabidopsis accessions were exposed to 2 h at 22°C or 35°C -UV-B to induce stomatal opening before transfer to 22°C or 35°C ± 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B for 3 h. Photon irradiance and treatment time were chosen as an effective UV-B dose to promote stomatal closure (Tossi et al., 2014). Stomatal apertures were measured as a ratio of width to length. Measurements were interpreted as the ability of UV-B to induce stomatal closure.

*Col-0 epidermal peels*

Stomatal aperture of Col-0 seedlings was found to differ significantly ($p<0.05$, one-way ANOVA) between 22°C and 35°C in the absence of UV-B (fig. 3.2.1a) in epidermal peels. Furthermore, stomatal aperture was significantly reduced after UV-B exposure at 35°C and 22°C ($p<0.05$, one-way ANOVA; fig. 3.2.1a). At 35°C +UV-B, stomatal aperture was reduced to very similar levels as observed for the 22°C -UV-B treatment group, and there was no statistically significant difference between these groups ($p>0.05$, one-way ANOVA).

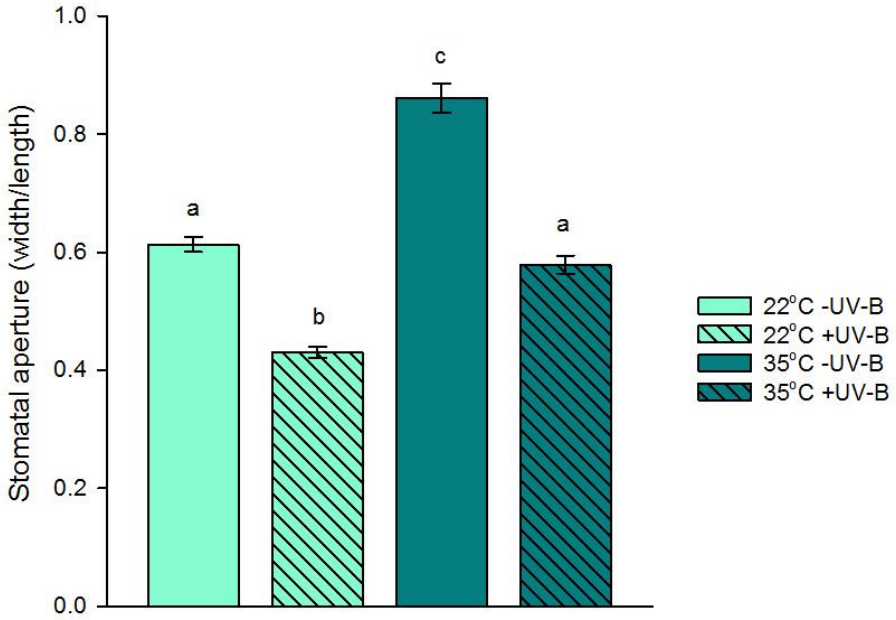


**Figure 3.2.1a. UV-B induced stomatal closure in Arabidopsis WT (Col-0) at 22°C and 35°C.** Epidermal peels from 4-5-week-old Arabidopsis (Col-0) leaves were incubated for 2 h at 22°C (light blue) or 35°C (dark blue) in the absence of UV-B to induce stomatal opening. Half of the strips at each temperature were transferred into 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B for 3 h. Stomatal aperture was measured by microscopy as a ratio of width-length. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA). n=9, error bars represent SE.

*Col-0 whole leaves*

To investigate whether UV-B perception of the mesophyll cells neighbouring the guard cells produces a systemic signal, affecting GC responses, whole leaves of 4-5 week old Arabidopsis (Col-0) plants were used for whole leaf bioassays. Two leaves from individual plants were exposed to the

experimental conditions. Leaves were peeled immediately post-exposure to produce epidermal peels for analysis. As figure 3.2.1b (green) shows, a decrease in stomatal aperture was seen after UV-B irradiation with the decrease at 35°C at a significant level ($p<0.05$, one-way ANOVA). The temperature response in the whole leaf was not found to be statistically significant ($p>0.05$, one-way ANOVA). The response in single epidermal peels (fig. 3.2.1b, blue) used as a treatment/buffer control in this experiment, showed a greater response to temperature ($p<0.05$, one-way ANOVA) and UV-B at 22°C ($p<0.05$, one-way ANOVA) than whole leaves. Whole leaves, particularly the ones exposed to 35°C ± UV-B wilted which encumbered peeling. As such, these data should be interpreted with caution.

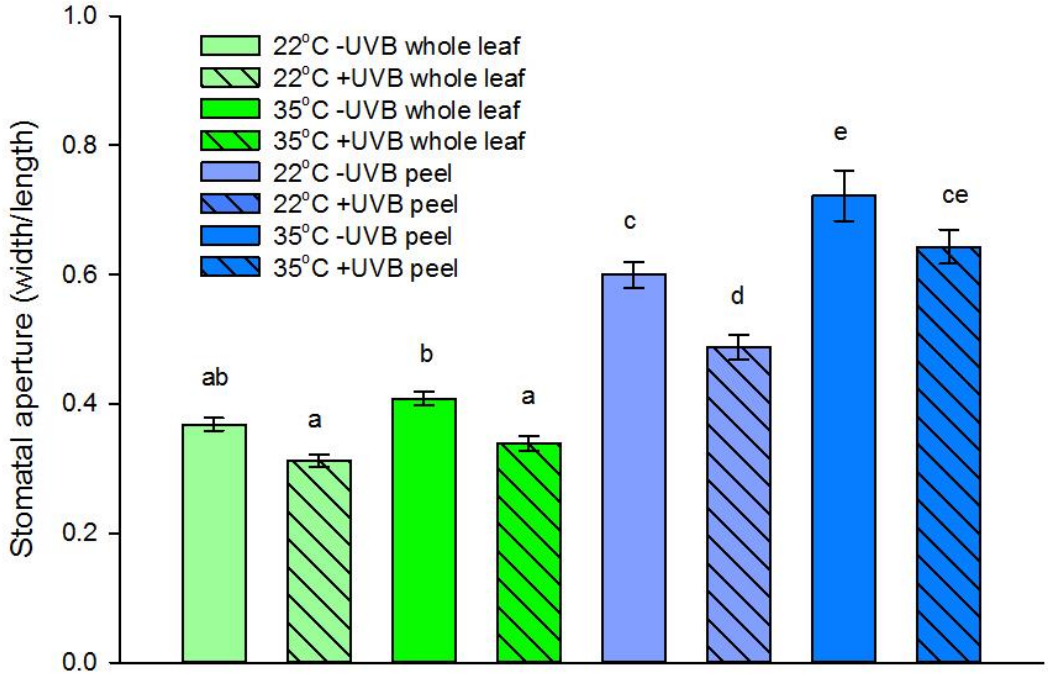


**Figure 3.2.1b. Stomatal aperture of whole leaves responded less to temperature and UV-B treatments than epidermal peels of Arabidopsis (Col-0)**. Whole leaves from 4-5-week-old Arabidopsis (Col-0) leaves were floated on MES/KCl buffer for 2 h at 22°C or 35°C to induce stomatal opening. Half of the leaves at each temperature were transferred into 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B and then peeled to produce epidermal strips. Stomatal aperture was measured by microscopy as a ratio of width-length. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA). Significant differences were only compared within separate methods. n=9 (whole leaves), n=3 (peels), error bars represent SE.

*Ws* and *Ler*

Wassilewskija and *Landsberg erecta* accessions of Arabidopsis showed reduced stomatal aperture in response to UV-B treatment at 22°C ($p<0.05$, one-way ANOVA; fig. 3.2.1c (a) and (b)). High temperature (35°C) failed to induce stomatal opening in Ws (fig. 3.2.1d) and *Ler* (data not shown). Stomata were almost completely closed, which prevented taking measurements (fig. 3.2.1d).

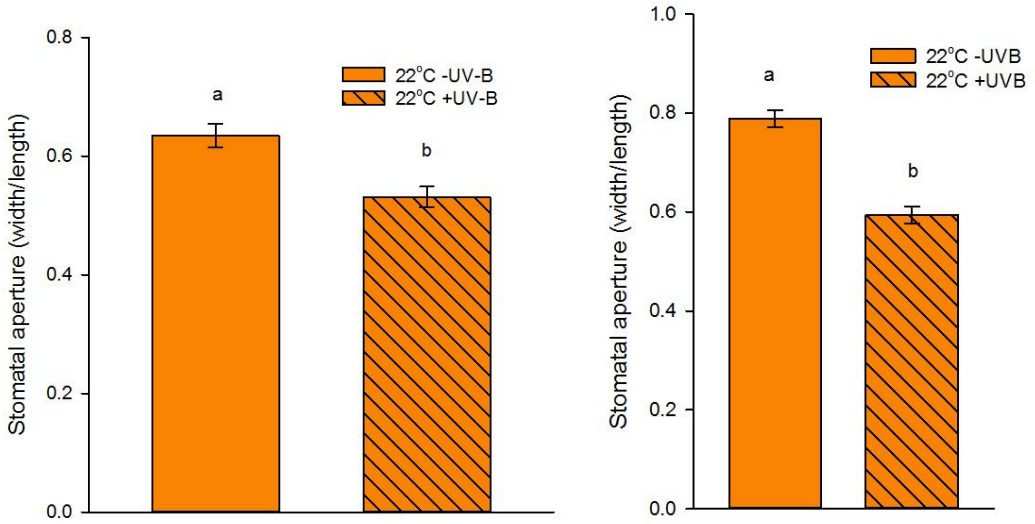


**Figure 3.2.1c. UV-B induced stomatal closure at 22°C in Arabidopsis (Ws and *Ler*)**. Epidermal peels from 4-5-week-old Arabidopsis leaves were floated on MES/KCl buffer for 2 h at 22°C (orange) or 35°C (data not shown) in the absence of UV-B to induce stomatal opening. Half of the strips at each temperature were then transferred into 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B with fresh buffer. Stomatal aperture was measured by microscopy as a ratio of width-length. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA), n=9, error bars represent SE. **(a)** Ws. **(b)** *Ler*.

**Ws 35°C -UV-B** **Ws 35°C -UV-B** **Ws 22°C -UV-B**

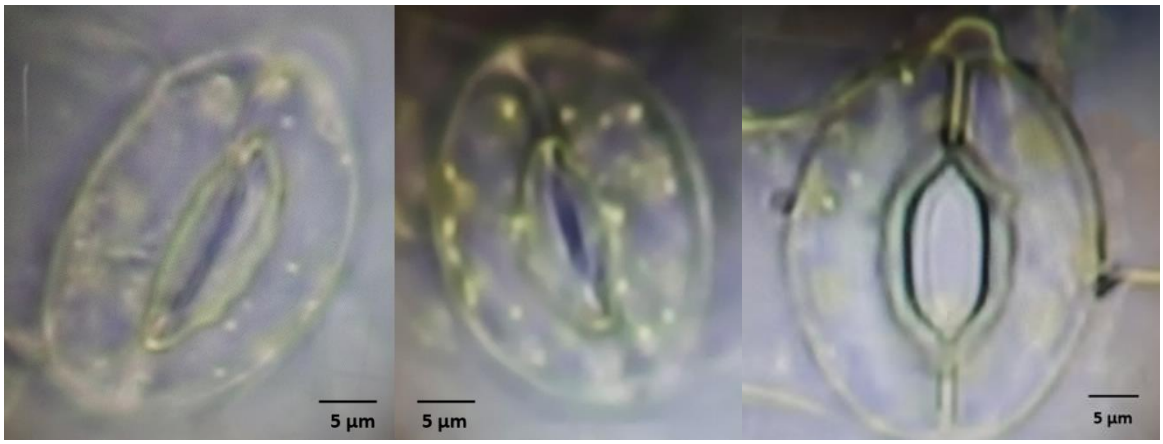


**Figure 3.2.1d. Stomata of Arabidopsis (accession Ws) subjected to 35°C or 22°C for 3 h without supplemented UV-B.** Epidermal peels of 4-5 week old Arabidopsis (Ws) leaves were floated on MES/KCl buffer for 2 h at 22°C or 35°C in the absence of UV-B to induce stomatal opening. The samples were then transferred to fresh buffer at the same temperatures for 3 h. Stomatal aperture was measured by microscopy as a ratio of width-length.

### **3.2.2** UVR8 mediates UV-B-induced stomatal closure at high temperature

Col-0 and *uvr8-6* were grown together to ensure identical exposure to growth and experimental conditions, and that plants were at the same developmental stage when leaves were harvested for peels. Similar to Col-0 controls, stomatal aperture was found to be significantly increased in *uvr8-6* null mutant at 35°C compared with 22°C in absence of UV-B ($p<0.05$, one-way ANOVA; fig. 3.2.2, green). In contrast to Col-0 controls, after UV-B exposure, neither the stomata of the 22°C treatment group or the 35°C treatment group of *uvr8-6* showed significantly altered stomatal aperture ($p>0.05$, one-way ANOVA; fig. 3.2.2, blue).

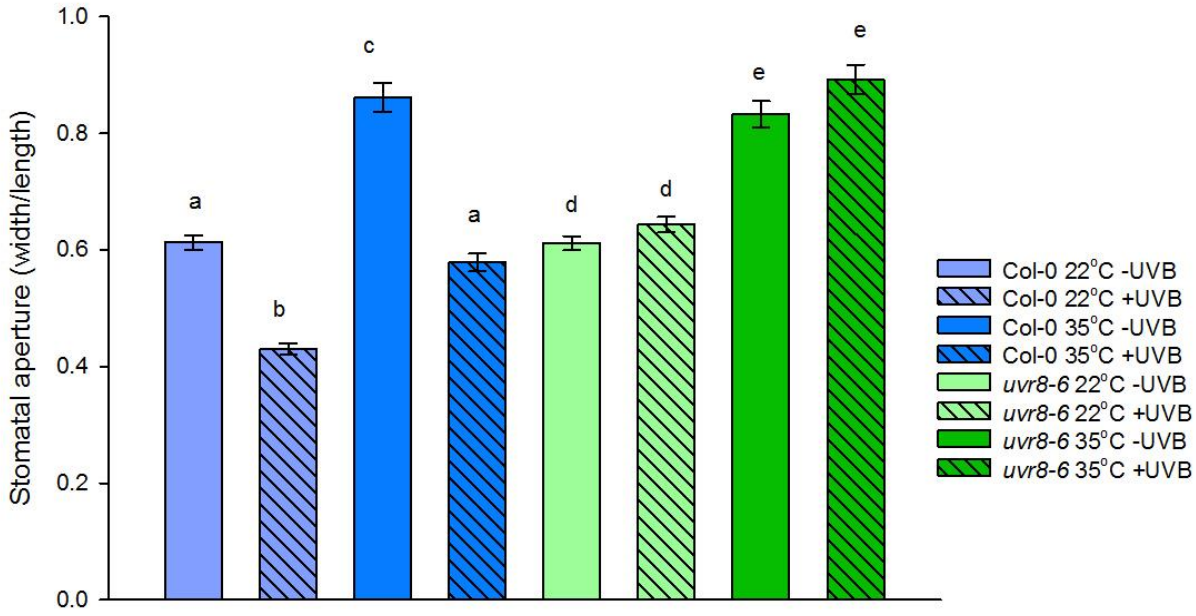


**Figure 3.2.2. UV-B did not reduce stomatal aperture in the Arabidopsis mutant *uvr8-6*.** Epidermal peels of 4-5-week-old Arabidopsis (Col-0 and *uvr8-6*) were incubated for 2 h at 22°C or 35°C in the absence of UV-B to induce stomatal opening. Half of the strips at each temperature were then transferred into 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B for 3 h. Stomatal aperture was measured by microscopy as a ratio of width-length. Blue = Col-0; green = *uvr8-6*. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA), n=9, error bars represent SE.

### **3.2.3** Guard cell viability

GC viability was measured in Col-0 and *uvr8-6* (fig. 3.2.3a) using FDA fluorescent probe. Epidermal peels of Col-0 and *uvr8-6* were subjected to 22°C or 35°C -UV-B for 2 h and then transferred to 22°C -UV-B (least stressful treatment) or 35°C +UV-B (most stressful treatment) for 3 h. Cells were incubated immediately post-exposure with 10 µm FDA for 10 min and then washed three times on fresh buffer to remove excess probe. Peels were examined by fluorescent microscope (excitation 470 nm) and micrographs analysed in ImageJ. GC viability for Col-0 was 71% after the 22°C -UV-B treatment and 72% after the 35°C +UV-B treatment (fig. 3.2.3a, green). GC viability in *uvr8-6* was 94% in the 22°C -UV-B treatment and 51% in 35°C +UV-B treatment (fig. 3.2.3a, orange). These data show that the combined treatment of high temperature and UV-B was only stressful to *uvr8* GCs which cannot produce sufficient protective pigments. Micrographs of epidermal peels showed live,

fluorescing GCs, and inviable, non-fluorescing GCs (fig. 3.2.3b). No viability tests were conducted on Ler and Ws, but micrographs examination showed GCs looked damaged at high temperature (fig. 3.2.1d (a) and (b)). GCs appeared viable at 22°C as they were not completely closed (fig. 3.2.1d (c)).

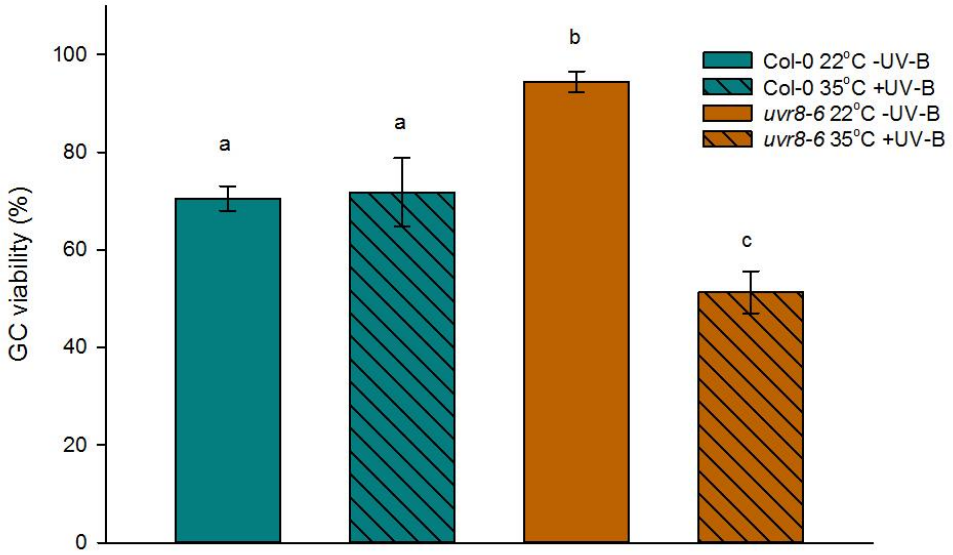


**Figure 3.2.3a. GC viability of Arabidopsis at 22°C -UV-B and 35°C +UV-B of Col-0 (blue) and *uvr8-6* (orange).** Epidermal peels were floated on MES/KCl buffer for 2 h at 22°C or 35°C in the absence of UV-B, and then exposed to 3 h 22°C -UV-B, or 35°C +UV-B. Post-treatment, cells were immediately washed with FDA - a stain only absorbed and metabolised by live cells. Peels were examined by fluorescent microscopy (excitation 470nm). Micrograph analysis was performed using ImageJ. Different letters indicate a statistically significant difference ($p<0.05$; arcsine transformation and one-way ANOVA) which were only compared within genotypes, error bars represent SE, n=7.

**(a)** **(b)**

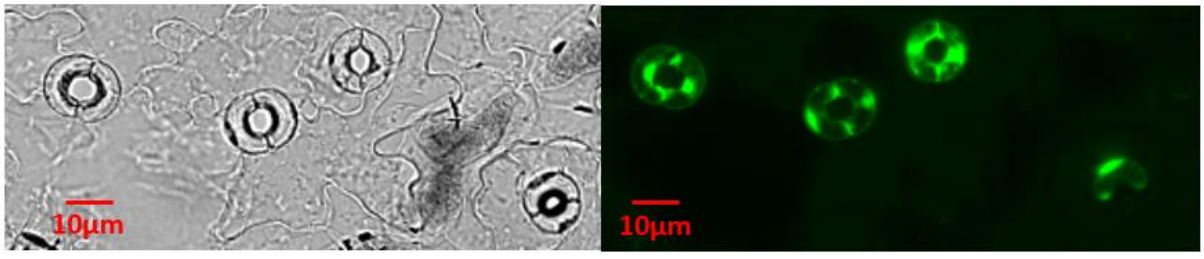


**Figure 3.2.3b. Eight GCs surrounded by pavement cells in an Arabidopsis epidermal peel.** Epidermal peels were incubated for 2 h at 22°C or 35°C in the absence of UV-B and then transferred to 22°C or 35°C ±UV-B for 3 h. Post-exposure, cells were immediately stained with FDA fluorescent probe. **(a)** FDA-stained cells. Image obtained by light microscopy. **(b)** FDA-stained cells. Image obtained by fluorescent microscopy (470 nm excitation). Images were processed in ImageJ.

### **3.2.4** ABA biosynthesis may not play a major role in short-term UV-B-induced stomatal closure at high temperature

The *nced3/5* mutant is in the Col-0 background. The *NCED* genes code for the enzyme 9-cis-epoxycarotenoid dioxygenase, required for ABA biosynthesis (Xiong and Zhu, 2003). The *nced3/5* mutant is deficient in ABA due to impaired biosynthesis. *nced3/5* showed significant stomatal opening in response to 35°C treatment compared with 22°C ($p<0.05$, one-way ANOVA; fig. 3.2.4) and a significant reduction in stomatal aperture in response to UV-B at both 22°C and 35°C ($p<0.05$, one-way ANOVA). Due to time and space restraints, this mutant was not grown simultaneously to its background line (Col-). Replication together with Col-0 would be needed to confirm the results.

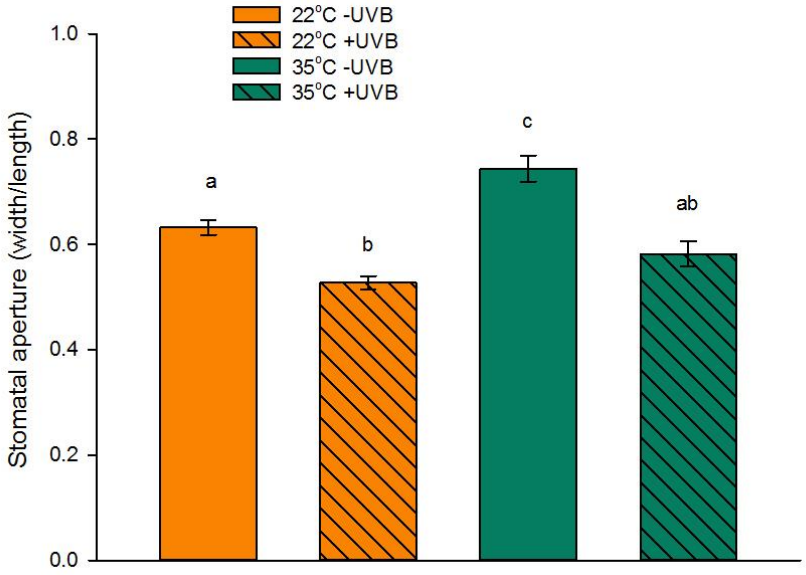


**Figure 3.2.4. UV-B induced stomatal closure at 22°C and 35°C in ABA-deficient Arabidopsis mutant *nced3/5*.** Epidermal peels of 4-5-week-old Arabidopsis *nced3/5* mutant were floated on MES/KCl buffer for 2 h at 22°C (orange) or 35°C (green) in the absence of UV-B to induce stomatal opening. Half of the strips at each temperature were then transferred into 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B for 3 h. Stomatal aperture was measured by microscopy as a ratio of width-length. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA), n=9, error bars represent SE.

### **3.2.5** UV-B induces NO accumulation in Arabidopsis

Epidermal peels from 5-week old Arabidopsis (Col-0 and *uvr8-6*) were prepared and subjected to experimental conditions as previously discussed. Post-exposure, peels were immediately incubated with 10 µm DAF-FM diacetate for 30 min. Peels were then washed on fresh buffer for 30 min, washed twice to remove excess probe, and then mounted on standard microscope slides for examination by fluorescent microscopy (excitation 470 nm). Micrograph images were analysed and NO quantified using ImageJ. Figure 2.3.5a shows the NO levels in Col-0 after temperature and UV-B treatment. There was a decrease in NO levels in 35°C compared with 22°C, however this difference was not statistically significant ($p>0.05$, one-way ANOVA). Furthermore, there was an increase in NO levels in both 22°C ($p<0.05$, one-way ANOVA) and 35°C ($p>0.05$, one-way ANOVA) after UV-B irradiation. In *uvr8-6* (fig. 2.3.5a) NO levels dropped in response to UV-B at 22°C, however not significantly ($p>0.05$, one-way ANOVA). At 35°C, NO levels did not change in response to UV-B treatment ($p>0.05$, one-way ANOVA). These data may suggest that UV-B increases NO levels in a UVR8-dependent manner. The alteration in NO levels coincide with alterations in stomatal aperture and may suggest that NO is involved in the closure response.

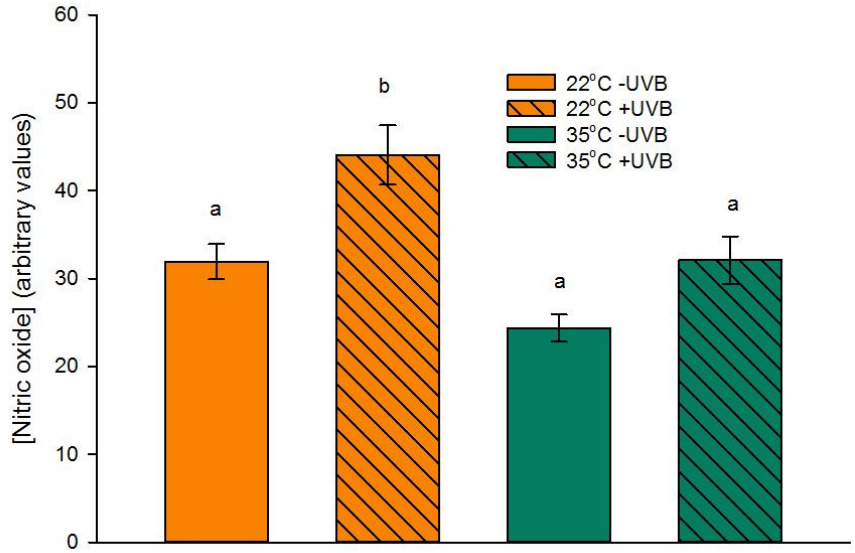


**Figure 3.2.5a. NO levels increased in response to UV-B irradiation in Arabidopsis WT (Col-0).** Epidermal peels from 4-week-old Arabidopsis (Col-0) leaves were subjected to 2 h pre-incubation at 22°C or 35°C in the absence of UV-B to induce stomatal opening and then transferred to 22°C or 35°C ±UV-B. Immediately post-exposure, the epidermal peels were incubated for 30 minutes with DAF-FM-DA, a probe for quantifying NO. Samples were examined by fluorescent microscopy (excitation 470 nm). Micrographs were analysed, and NO quantified in ImageJ. Fluorescence was described as arbitrary values. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA), n=8, error bars represent SE.

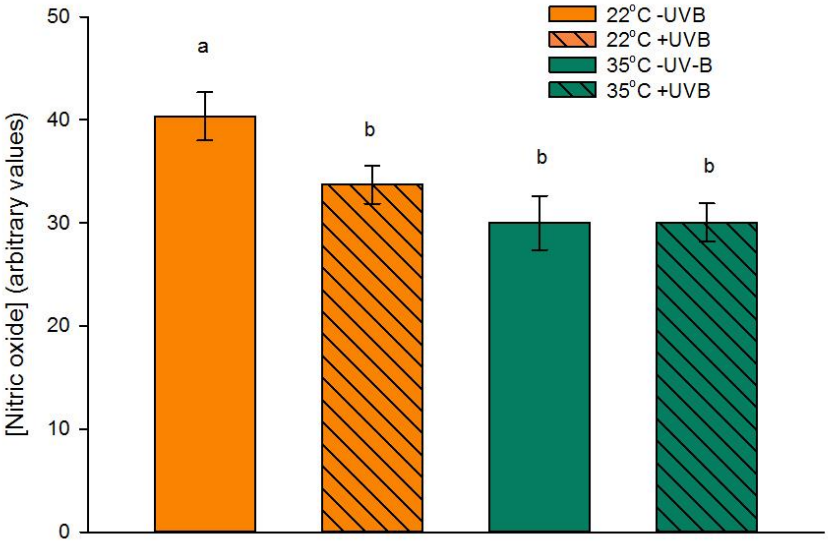


**Figure 3.2.5b. NO levels did not increase in response to UV-B irradiation in the Arabidopsis *uvr8-6* mutant.** Epidermal peels from 4-week old Arabidopsis (*uvr8-6*) leaves were subjected to 2 h pre-incubation at 22°C or 35°C in the absence of UV-B to induce stomatal opening and then transferred to 22°C or 35°C ±UV-B. Immediately post-exposure, the epidermal peels were incubated for 30 minutes with DAF-FM-DA, a probe for quantifying NO. Samples were examined by fluorescent microscopy (excitation 470 nm). Micrographs were analysed, and NO quantified in ImageJ. Fluorescence was described as arbitrary values. Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA), n=8, error bars represent SE.

## 3.3 Discussion

Stomatal aperture has been shown to be influenced by various environmental conditions, such as light and water availability (Kollist et al., 2014). Here, it was tested how UV-B impacts high temperature-induced stomatal opening by subjecting epidermal peels or whole leaves of Arabidopsis to 22°C or 35°C ±UV-B. NO accumulation and GC viability were also investigated.

Stomatal movement was measured in three different accessions of Arabidopsis. Stomatal opening in response to elevated temperature has previously been described in the literature (Rogers et al., 1979; Hofstra and Hesketh, 1969; Feller, 2006; Urban et al., 2017). High temperature-mediated stomatal opening was seen here in the Arabidopsis ecotype Col-0. In the accessions *Ler* and Ws no high temperature response was observed. This is likely due to high cellular stress and/or cell death rather than a lack of response. Examination of epidermal peels showed that GCs were completely closed after high temperature treatment (fig. 3.2.1d), indicating stress. Due to time constraints of the study, further adjustments of experimental temperature conditions could not be explored. Viability staining using FDA could confirm the suspected cell death of Ws and *Ler* at 35°C. It is possible that a slightly lower temperature, for example 28°C, would have produced an opening response equivalent to that seen in Col-0 at 35°C. Differences in heat tolerance could result from the natural habitat in which the different accessions have evolved. Ws evolved in Belarus, *Ler* in Germany, and Col-0 in Columbia in the US (TAIR, 2018). Tossi et al. (2014) performed bioassay experiments at 25°C and exposed *Ler* to UV-B levels higher than those used in this study, maintaining a GC viability of approximately 70%. This indicates that the temperature, or a combination of high temperature and UV-B irradiation is detrimental to the GCs of this accession. It is important to select temperatures within a range that does not subject seedlings to stress, or to select a suitable accession based on experimental conditions.

UV-B supplementation induced stomatal closure in Col-0 at both 22°C and 35°C (fig. 3.2.1a). These results are similar to those obtained by Tossi et al. (2014) where stomata of *Ler* were seen to close in response to 5.46 µmol $m^{-2}$ $s^{-1}$ UV-B. A lower UV-B fluence rate was used (2.7 µmol $m^{-2}$ $s^{-1}$) here to more accurately reflect strong sunlight and reduce the risk of plants suffering stress. Low fluence rate UV-B is considered 0.1-1 µmol $m^{-2}$ $s^{-1}$, with >1 µmol $m^{-2}$ $s^{-1}$ approaching stress level (Tong et al., 2008). Using 2.7 µmol $m^{-2}$ $s^{-1}$ was a compromise between a low enough fluence rate to not cause excessive stress, and still induce significant stomatal closure. Stomatal aperture is adjusted to maintain a balance between efficient $CO_2$ uptake and water preservation (Hassidim et al., 2017). At high temperatures plants are at risk of heat stress. In well-watered conditions, allow stomata to remain open so that evapotranspiration can occur, which results in leaf cooling and likely mitigates heat stress (Crawford et al., 2012). UV-B appears to counteract this crucial cooling mechanism, possibly to better balance cooling capacity and water status in dynamic conditions. This may also be a pre-cautionary response to anticipated UV-B stress where resources and metabolic efforts are relocated from photosynthesis and growth, as seen in thermomorphogenesis, to pigment and antioxidant production.

Stomata on whole leaf samples showed less GC movement than the epidermal peel control. Of note here is that limited time restricted both further repeats and method development for this experiment. Leaves exposed to 35°C wilted, which hampered peeling. This may have damaged GCs and affected results. Additionally, leaves were floated on the opening buffer and could not be submerged. This is unlikely to have affected results extensively as sufficient $K^+$ ions should be available from the within the leaves to induce and maintain closure. However, to be consistent with

the experiments using peels, where the epidermis was in constant contact with the buffer, and to be certain sufficient $K^+$ is available for closure, submersion of leaves into the buffer should be investigated. A shorter exposure time may also allow peeling before leaves wilt. Alternatively, the results obtained here may point towards inter-cell-layer communication. It may be that the striking effect that both temperature and UV-B appear to have in epidermal peels is diminished when the cells are in contact with the mesophyll. Several signals could be transmitted between the mesophyll and GCs, coordinating stomatal behaviour (Lawson et al., 2014). For example, Roelfsema et al. (2006) noted that photosynthetically active radiation (PAR)- induced stomatal responses did not occur in albino leaves of *Vicia faba* where GCs lacked chloroplasts and neither in *Chlorophytum comosum*, where chloroplasts were present in GCs. These observations suggest that photosynthesis in the mesophyll tissue may be important for stomatal movement in response to PAR. The results presented here suggest an involvement of the mesophyll in stomatal closure, but further repeats and methods adjustments are required to confirm these results. It is also possible that pigments in the leaves work to diffuse and mitigate the incoming UV-B radiation (Caldwell et al., 1983; Christie and Jenkins, 1996), lessening the signal that reaches the GCs, and thereby reducing the response.

In this study, a UVR8 null mutant, *uvr8-6* (Col-0 background), was used to elucidate the involvement of the UV-B photoreceptor UVR8 in UV-B-induced stomatal closure responses. As was shown in fig. 3.2.2, elevated temperature induced stomatal opening in this mutant, in the absence of UV-B; however, UV-B irradiation failed to induce stomatal closure at both 22°C or 35°C. Tossi et al. (2014) showed the involvement of UVR8 in stomatal closure at 22°C. Here, UVR8 was shown to also mediate the stomatal closure response at 35°C. This further indicates that the response seen here is a photomorphogenic response that occurs below the stress level and does not involve UVR8-independent signalling. Very little evidence exists in the literature regarding high temperature signalling in stomatal movement, encumbering inquiries into the signalling pathways involved in this response. It would be interesting to examine the interface of crosstalk between the UV-B- and high temperature-signalling pathways, perhaps by investigating the effect of UV-B on typical high temperature-induced transcripts such as *HSP70* (Kumar and Wigge, 2010).

High temperatures and high UV-B often occur simultaneously with drought. ABA mediates stomatal closure in response to drought and reduced RH of ambient air (Daszkowska and Szarajeko, 2013). Tossi et al. (2014) suggested that part of the UV-B-induced stomatal closure response may be mediated by ABA. They previously showed that ABA increased by 100% in UV-B irradiated *Zea mays* leaves, indicating a role for ABA in UV-B responses and acclimation (Tossi et al., 2009). Here the ABA-deficient mutant, *nced3/5*, showed a similar closure response to the Col-0 WT post-UV-B treatment. This indicated that despite ABA biosynthesis being impaired, stomata were capable of responding to UV-B. This may suggest that short-term UV-B-induced stomatal closure likely does not involve ABA, but the role of ABA in maintained stomatal closure in response to UV-B was not investigated here and can therefore not be ruled out.

NO has been shown to be closely interlinked with ABA signalling (Neill et al., 2008). Its action is, however, complex. Wilson et al. (2009) showed that ABA signalling must be partly functional for effective NO signalling. The results obtained here point towards a less central role for ABA in the NO-mediated stomatal closure response. It is important to highlight the use of an ABA-deficient mutant which is impaired in ABA biosynthesis, but not completely deficient in ABA. It may be that an intact ABA signalling pathway contributes to UV-B-induced and NO-mediated stomatal closure but is not essential. It would be interesting to investigate NO levels in the *nced3/5* mutant. NO levels were investigated at 22°C and 35°C ± UV-B in Col-0 (WT) and the *uvr8-6* mutant using a fluorescent probe. NO has been shown to mediate stomatal closure in Arabidopsis at 22°C in response to UV-B (Tossi et

al., 2014). This was observed in Col-0 in this study (fig. 3.2.5a). An increase in NO levels was observed in response to UV-B at 35°C, correlating with stomatal closure (fig. 3.2.5a; fig. 3.2.1a). Interestingly, the highest NO levels occurred in 22°C +UV-B and the lowest NO concentration at 35°C –UV-B. Reduced NO in the 35°C -UV-B treatment group correlates with high temperature-induced stomatal opening. This provides a novel potential component in the high temperature-induced stomatal opening response. It would be interesting to investigate the stomatal aperture response in a mutant deficient in NO, or after the application of a NO scavenger. NO levels in the *uvr8-6* mutant were also highest at 22°C –UV-B, with reduced NO seen in 35°C –UV-B (fig. 3.2.5b). This dismisses the involvement of UVR8 in a potential NO-mediated stomatal opening mechanism at 35°C. UV-B treatment did not alter NO levels significantly at 22°C or 35°C in *uvr8-6*. This suggested that the rise in NO levels in response to UV-B seen in the WT is UVR8-mediated. This could explain the lack of stomatal closure seen in *uvr8-6* in response to UV-B (fig. 3.2.2).

GC viability is crucial to meaningful results. Here, the viability of GCs in epidermal peels of Col-0 WT exposed to 35°C in the presence of UV-B (deemed the most stressful treatment) was similar to the viability of GCs exposed to the least stressful treatment of 22°C -UV-B. Viability was calculated to approximately 70% for both treatments (fig. 3.2.3a). 70% viability was considered sufficient for continued experiments using the same UV-B and temperature treatments. Unpublished work on stomatal opening in response to high temperature conducted at the department prior to this study indicates that maximum stomatal opening is obtained in Col-0 at 35°C (Kostaki et al., unpublished), however, it could be beneficial to explore lower temperatures still considered “high” in order to maximise GC viability. GC viability for the *uvr8-6* mutant were 94% in the least stressful treatment and 51% in the most stressful treatment (fig. 3.2.3a). It would be interesting to investigate the viability of GCs in all conditions to see whether the effects of UV-B and high temperature are additive in this response.

# CHAPTER 4: UV-B ALTERS STOMATAL DENSITY AT 35°C AND 22°C

## 4.1 Introduction

Stomata serve as the interface of plant-atmosphere gas and water exchange. In addition to adjusting stomatal aperture, long-term stomatal adaptations to different environmental conditions are achieved by altering the number of stomata on leaves. Stomata develop in a tightly regulated manner in young leaves (Pillitteri and Dong, 2013). Stomatal development is influenced by environmental signals and coordinated by a set of genes, including *SPCH*, *MUTE* and *FMA* (fig. 1.1.2). High temperature conditions increase the need for leaf cooling. As stomata are the channels for cooling by evapotranspiration, temperature shifts influence stomatal development. High temperature has been shown to have differing effects on stomatal density. A decrease was seen in Arabidopsis (Crawford et al., 2012; *Lau* et al., 2018), but an increase was seen in soy bean (Jumrani et al., 2017). Lau et al. (2018) showed that high temperature also reduces stomatal index via PIF4-mediated suppression of *SPCH*.

UV-B has been shown to cause varied effects on stomatal development. Both decreased (Dai et al., 1995) and increased (Kostina et al., 2001) stomatal densities have been observed post UV-B irradiation. Additionally, inconclusive results have also been reported. UV-B reduced adaxial stomatal density in two out of four lines of soy bean but failed to reduce abaxial stomatal density in all but one line (Gitz III et al., 2005). Wargent et al. (2009) showed that UV-B decreased stomatal index in Arabidopsis (*Ler*) and that this response was UVR8-mediated. Hectors et al. (2010) showed there was no difference in stomatal index in response to UV-B in the Col-0 accession of Arabidopsis. Evidence from the literature is inconclusive concerning the separate effects of high temperature and UV-B signalling on stomatal development. Here, these two signals were investigated separately and in unison for any combined effects that may enlighten the long term effects on stomatal development by these two environmental factors.

## 4.2 Results

### **4.2.1** UV-B reduces stomatal density via a UVR8-independent mechanism

Stomatal density was measured in Arabidopsis (Col-0 and *uvr8-6*). Plants were grown until maturity, which was approximately 6-7 weeks. Leaf impressions were produced using the dental resin and nail varnish method as described in chapter 2. Growth conditions were 22°C and 28°C ± UV-B. Leaf impressions were analysed by light microscopy and stomatal density was calculated as number stomata per unit area (40x40 µm). High temperature decreased stomatal density in both Col-0 and *uvr8-6* but not to a significant degree ($p>0.05$, one-way ANOVA; fig. 4.2.1). The stomatal density of Col-0 was not significantly altered in 22°C or 28°C in response to UV-B ($p>0.05$, one-way ANOVA; fig. 4.2.1, yellow). There was however a small increase after UV-B irradiation at 22°C, and a decrease at 28°C. The *uvr8-6* mutant displayed a reduction in stomatal density in both the 22°C and 28°C +UV-B treatments compared with the non UV-B control, both to a significant level ($p<0.05$, one-way ANOVA, fig. 4.2.1, green).

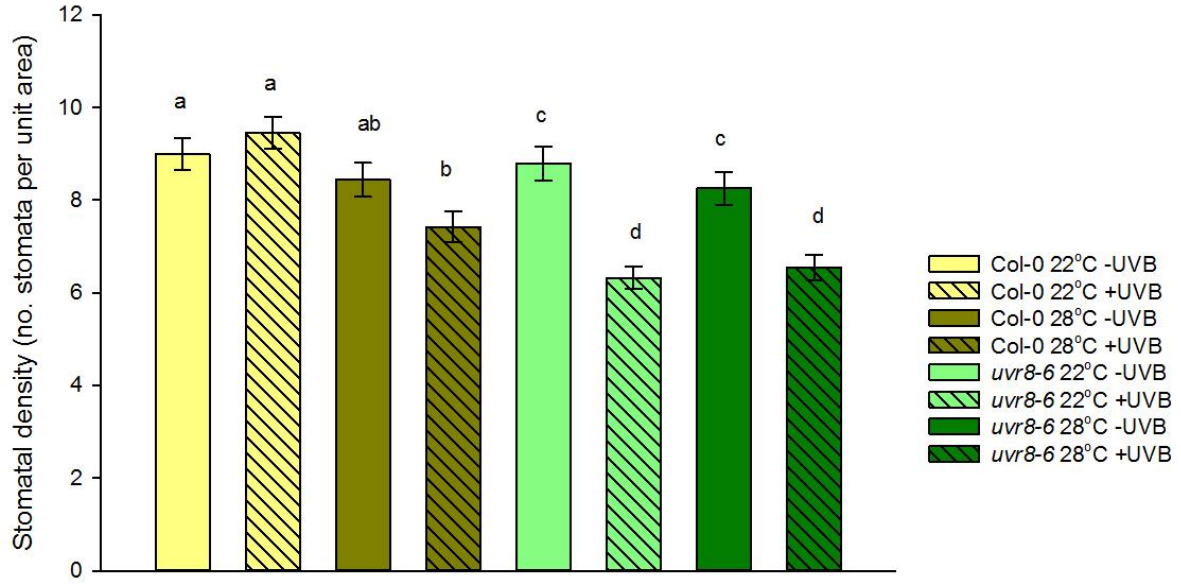


**Figure 4.2.1. UV-B does not significantly alter stomatal density in Col-0 but does so in *uvr8-6*.** Arabidopsis were germinated at 22°C in the absence of UV-B and then randomly allocated to 22°C or 28°C ±UV-B. Leaves from the middle rosette that developed completely in the experimental conditions were harvested at the first bolt emergence (4-7 weeks, dependent on the treatment). Leaf impressions were and examined by light microscopy. Stomatal density was calculated as the number of stomata in a given area (40x40 µm). Different letters indicate a statistically significant difference ($p<0.05$, one-way ANOVA) which were only compared within genotypes. n=48, error bars represent SE.

### **4.2.2** UV-B does not affect stomatal index in the Col-0 accession of Arabidopsis

Stomatal index was measured in Arabidopsis (Col-0 and *uvr8-6*). Plants were grown until maturity and harvested on the day of the first bolt emergence, approximately 6-7 weeks. Leaf impressions were produced using the dental resin and nail varnish method, as described in chapter 2. Growth conditions were 22°C and 28°C ± UV-B. Leaf impressions were analysed by light microscopy and stomatal index was calculated as the number of stomata divided by the total number epidermal cells within a given area (40x40µm) plus stomata. No statistically significant differences in stomatal index was observed in response to temperature treatment in Col-0 or *uvr8-6* ($p>0.05$, one-way ANOVA, fig. 4.2.2). Neither was there a significant difference in stomatal index in response to UV-B in Col-0, regardless of temperature ($p>0.05$, one-way ANOVA, fig. 4.2.2, yellow). In the *uvr8-6* mutant, stomatal index increased significantly in response to UV-B at 28°C ($p<0.05$, one-way ANOVA, fig. 4.2.2, green).

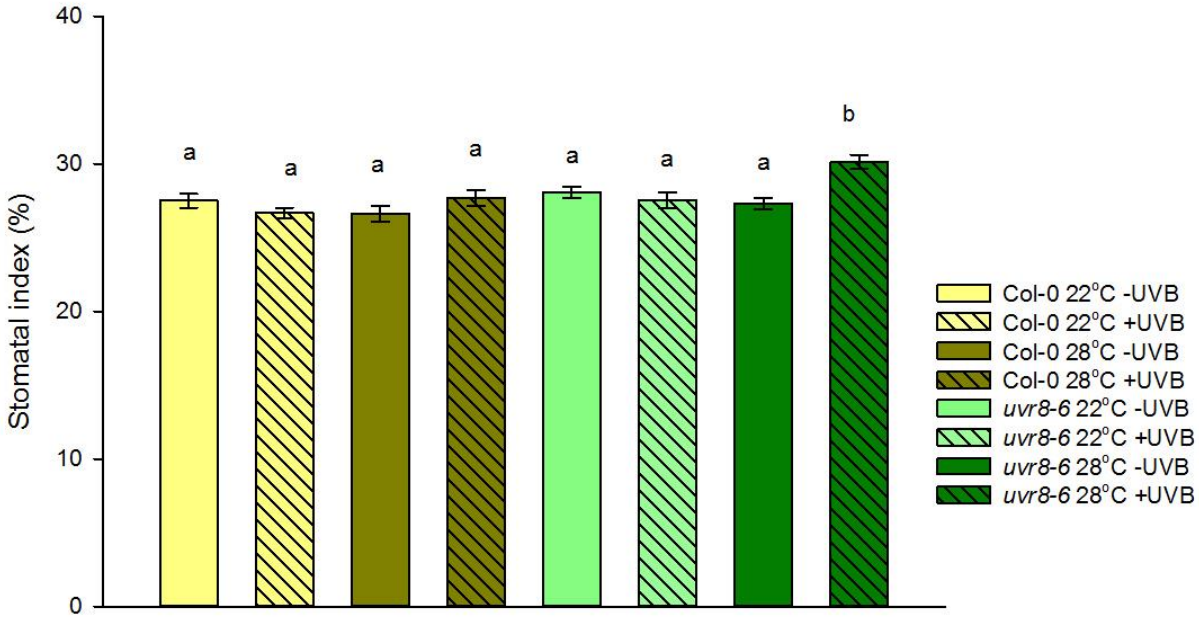


**Figure 4.2.2. UV-B does not significantly alter stomatal index in Col-0.** Arabidopsis were germinated at 22°C in the absence of UV-B and then randomly allocated to 22°C or 28°C ±UV-B. Leaves from the middle rosette were harvested at the first bolt emergence (4-7 weeks, dependent on the treatment). Leaf impressions were produced and examined by light microscopy. Stomatal index was calculated as the number of stomata divided by the total number epidermal cells, plus stomata, in a given area (40x40 µm). Different letters indicate a statistically significant difference ($p<0.05$; arcsine transformation and one-way ANOVA) which were only compared within genotypes. n=48 error bars represent SE.

### **4.2.3** UV-B alters plant morphology

Plants exposed to UV-B showed delayed flowering, of approximately 1 week, compared with non-irradiated plants. Plants of both Col-0 and *uvr8-6* exposed to 28°C -UV-B bolted at exactly 5 weeks, however the Col-0 plants exposed to 28°C +UV-B plants did not bolt until week 6. The *uvr8-6* mutant receiving the same treatment bolted at 5 weeks and 3 days. Both the Col-0 and *uvr8-6* plants exposed to 22°C -UV-B bolted at 6 weeks and 2 days. The *uvr8-6* plants exposed to 22°C +UV-B bolted at 6 weeks and 4 days. The Col-0 plants exposed to the same treatment had not bolted at week 7 which is when they were harvested due to experimental time constraints.

In Col-0, high temperature induced petiole elongation (fig. 4.2.3a (c)). This elongation was abolished by UV-B treatment (fig. 4.2.3a (d)), consistent with the role of UV-B in thermomorphogenesis inhibition (Hayes et al., 2017). UV-B also reduced petiole length at 22°C in Col-0 (fig. 4.2.3a (b)). In *uvr8-6*, petiole length increased in response to high temperature (fig. 4.2.3b (c)). UV-B failed to inhibit petiole elongation at 22°C and 28°C (fig. 4.2.3b (b) and (d)) in *uvr8-6*, confirming a role for UVR8 in this response. No UV-B-induced changes in leaf colouration were observed in Col-0 (fig. 4.2.3a). In *uvr8-6*, leaf chlorosis was observed in both the 22°C (fig. 4.2.3b (a) and (b)) and the 35°C (fig. 4.2.3b (c) and (d)) treatment groups which was exacerbated by UV-B irradiation (fig. 4.2.3b (b) and (d)). These results were interpreted as indicators of plant perception of the experimental conditions.


**Figure 4.2.3a. Mature WT (Col-0) Arabidopsis plants grown in different temperature and light conditions. (a)** 22$^{o}$C -UV-B. **(b)** 22$^{o}$C +UV-B. **(c)** 28$^{o}$C -UVB **(d)** 28$^{o}$C +UV-B**.** Images obtained with Nikon D3200 and processed in GIMP 2.

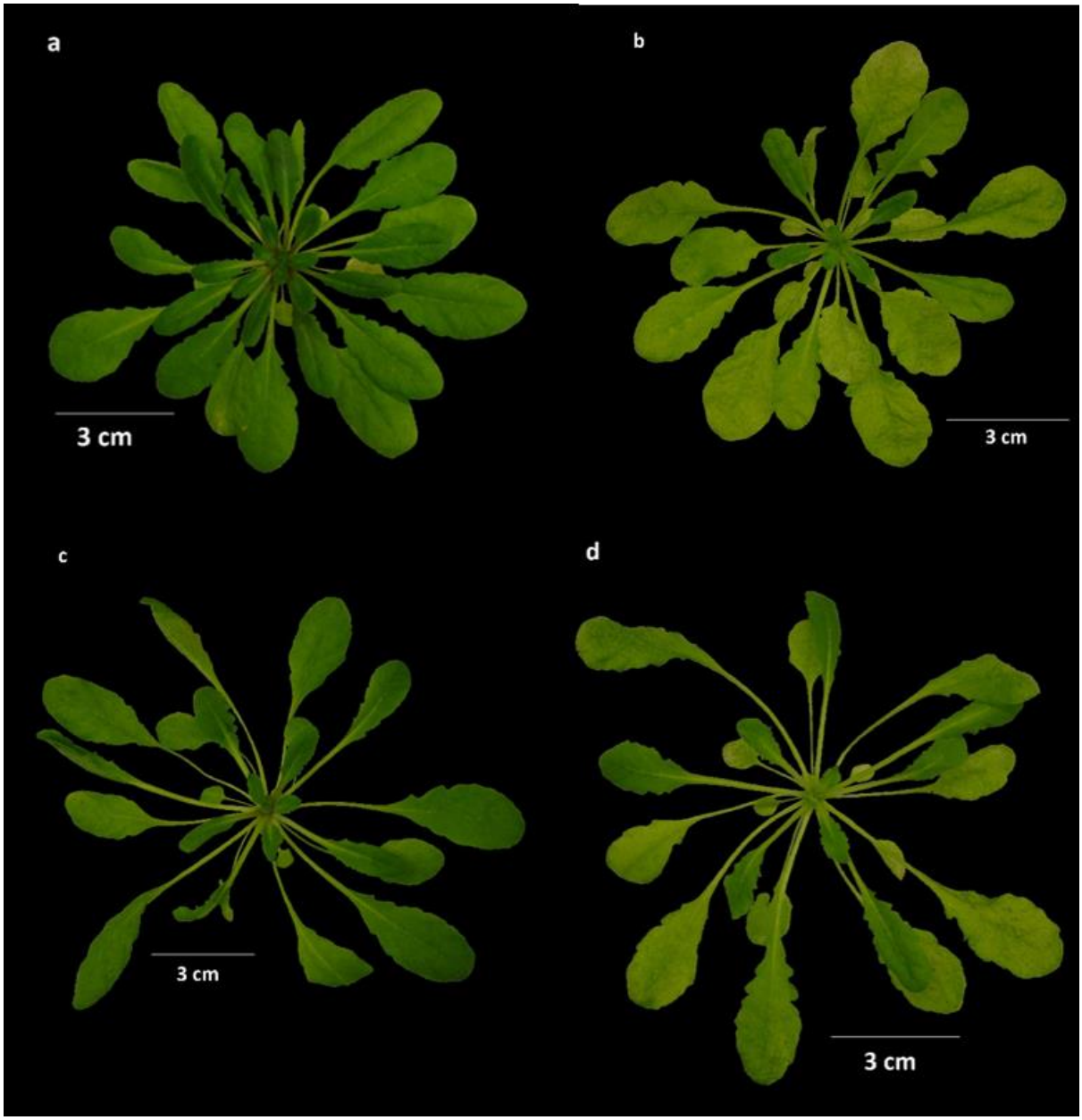


**Figure 4.2.3b. Mature Arabidopsis (*uvr8-6*) plants grown in different temperature and light conditions. (a)** 22°C -UV-B. **(b)** 22°C +UV-B. **(c)** 28°C -UVB **(d)** 28°C +UV-B**.** Images obtained with Nikon D3200 and processed in GIMP 2.

## 4.3 Discussion

Stomatal density was lower at higher temperature in both WT and *uvr8-6*. This is in accordance with the literature where a reduction was seen in several species of plant (Beerling and Chaloner, 1993; Luomala et al., 2005; Crawford et al., 2012; Lau et al., 2018). The opposite effect has been observed in soy bean (Jumrani et al., 2017). This indicates that stomatal density is highly dynamic, and responses can vary greatly between species and likely depends on what temperature range is considered "high" for specific species. Stomatal density was shown to reduce in response to high levels of UV-B in rice (Dai et al., 1995). In soy bean, UV-B reduced abaxial stomatal density in one line

but failed to produce the same result in two other lines (Gitz et al., 2005). Conversely, stomatal density was increased in response to UV-B in birch seedlings (Kostina et al., 2001). Here, stomatal density was increased in response to UV-B at 22$^{o}$C but reduced after UV-B irradiation at 35$^{o}$C. Neither of these effects were found to be significant compared with their non-UV-B-treated controls. Because this study was considered preliminary, further replication is encouraged to elucidate whether this is a true trend. Interestingly, stomatal density in *uvr8-6* was significantly reduced after UV-B irradiation at both 22$^{o}$C and 28$^{o}$C. These data may suggest that stomatal density is reduced by a UVR8-independent mechanism but increased in a UVR8-mediated manner. In WT plants, the two pathways would be in balance but in the mutant, the independent pathway would dominate thus reducing stomatal density. It would be interesting to investigate the response in a constitutively active UVR8 line, such as the UVR8$^{W285A}$ mutant (Heijde et al., 2013).

No difference in stomatal index was observed following high temperature or UV-B treatment in Col-0, in our experimental conditions. Lau et al. (2018) saw a reduction in stomatal index in response to high temperature (28$^{o}$C). The same study used both long (16 h light, 8 h dark) and short (8 h L, 16 h dark) photoperiods. Short photoperiods were observed to amplify the temperature-response. Here, 10 h photoperiods were used, which may explain the lack of reduction in stomatal index seen in Col-0 in response to high temperature. The Lau et al. study also used mature cotyledons (27 days old post germination), rather than mature mid-rosette leaf which may also account for the differences between the studies. A UVR8-mediated increase in stomatal index was observed in Arabidopsis grown in 10 h photoperiods with diurnally varying UV-B fluence rates (Wargent et al., 2009). Conversely, Hectors *et al.* (2010) saw no change in stomatal index in Arabidopsis plants grown in 10 h photoperiods with a low UV-B fluence rate applied for 2 h daily. These observations could be indicative of a strong influence of temperature range, photoperiod and UV-B dosage on the stomatal development response. The increase in stomatal index seen here in response to UV-B at 28$^{o}$C in *uvr8-6* could indicate that there is a temperature-dependent and UVR8-independent response to UV-B. The lack of a similar response in Col-0, however, suggests this result may be an anomaly, or that UVR8 acts as a suppressor of the response at high temperature. Further replicates are required to confirm this.

The results presented here may suggest that the UV-B and high temperature treatments used have stronger effect on epidermal cell expansion than development of the stomatal lineage. UV-B has been seen to reduce leaf size in various plant species, for example lettuce (Wargent et al., 2009), soy bean (Gitz III et al., 2005), pea plants (Nogués et al., 1998), maize (Fina et al., 2017) and Arabidopsis (Hayes et al., 2017). Reduced leaf size was found to result from reduced epidermal cell expansion in Arabidopsis (Hectors et al., 2010), possibly due to changes in the cell wall composition (Jacques et al., 2011). Alternatively, it was shown by Nogues et al. (1999) to result from a reduction in the number of epidermal cells. Wargent et al. (2009) showed that UV-B inhibits epidermal cell division and that this response is independent of UVR8. Epidermal cell expansion was increased in a WT Arabidopsis plant in response to UV-B, but not in a *uvr8* mutant, indicating a UVR8-dependent mechanism for this response. The same study showed a pronounced reduction in stomatal density in a *uvr8* mutant compared to the WT, similar to the results presented here.

Visual examination of plants subjected to the development experimental conditions showed clear morphogenic responses to high temperature and UV-B (fig. 4.2.3a and 4.2.3b). High temperature-induced thermomorphogenesis in mature rosettes is prominently characterised by elongated petioles, as shown in fig. 4.2.3a (c) and 4.2.3b (c). This response is mediated by the bHLH PIF4, as mutants deficient in PIF4 do not exhibit hypocotyl elongation at high temperature (Koini et al., 2009); a phenotype reminiscing the phenotype of the Arabidopsis WT displayed in fig. 4.2.3a (d). It

was later shown that PIF4 regulates auxin levels and that this in turn regulates growth (Franklin et al., 2011). Hayes et al. (2017) showed that UV-B inhibits thermomorphogenesis, via PIF4, and that this response involves UVR8. Similar phenotypes were observed in this study (fig. 4.2.3a and 4.2.3b), where UV-B abolished the thermomorphogenic effect in Col-0 but not in *uvr8-6*. These results suggest that the plants perceived the environmental signals intended and that any changes in stomatal density and index were indeed true responses to these conditions.

# CHAPTER 5: DISCUSSION

## 5.1 Stomatal aperture

High temperature-induced stomatal opening and UV-B-induced stomatal closure (at 22°C) were observed in this study, consistent with previous findings (Rogers et al., 1979; Hofstra and Hesketh, 1969; Feller, 2006; Urban et al., 2017; Nogues et al., 1999; Tossi et al., 2014; Dai et al., 1995; He et al., 2013). An understanding of the combined effects of these signals on stomatal aperture is lacking. Here it was shown that UV-B antagonises the high temperature-induced stomatal opening response (fig. 3.2.1a), mediating stomatal closure at 35°C. The UV-B-induced closure response was greater at 35°C than at 22°C, suggesting that increased apertures at 35°C provided a greater opportunity to visualise UV-B-induced closure. Stomata of intact leaves showed reduced stomatal aperture in all treatments (fig. 3.2.1b). Additionally, the differences between the treatments were less than in the epidermal peels (fig. 3.2.1b). This may suggest extensive inter-cell layer communication and moderation of temperature responses as well as mitigation of UV-B effects by mesophyll pigment production. Vanhaelewyn et al. (2016) point out that stress responses occur in non-acclimated plants at high doses of UV-B. Because the lab-grown seedlings used in this study were not exposed to UV-B prior to the experiments, they were not acclimated to UV-B. This could mean they respond differently than do acclimated plants. It would therefore be interesting to study stomatal responses in plants pre-treated with UV-B.

Limited information exists on the signalling components involved in both high temperature-induced stomatal opening, and UV-B-induced stomatal closure. It was shown by Tossi et al. (2014) that the UV-B photoreceptor, UVR8, mediates the stomatal closure response at 22°C in Arabidopsis. The results obtained here confirm this. In addition, our data suggest a role for UVR8 in the prevention of high temperature-mediated stomatal opening as the closure response was not significant in the *uvr8* mutant (fig. 3.2.2). It is therefore possible that UV-B, perceived by UVR8, acts as a brake on high temperature-induced stomatal opening, in addition to its inhibitory effect on thermomorphogenesis. Downstream of the UVR8 photoreceptor, COP1 and HY5/HYH were shown to be involved in the UV-B mediated stomatal closure response at 22°C (Tossi et al., 2014). HY5 and HYH are transcription factors; their involvement in stomatal closure indicates altered gene expression in response to UV-B. Tossi et al. (2014) recorded that stomata started to close within 30 min of commencing UV-B irradiation but that maximum closure was seen 3 h after first exposure. It is possible that the initial closure response is mediated by a mechanism not involving gene expression, but maintenance of closure and greater closure requires altered gene expression, as the suggested signalling pathway in figure 5.1 depicts. In the same study, the authors suggested ABA involvement in the closure response as ABA accumulation has been observed post-UV-B irradiation in Arabidopsis (Tossi et al., 2009). ABA can be released from conjugation with other molecules (Dietz et al., 2000), instead of *de novo* synthesis, something that would not require altered gene expression. Here, apertures of *nced3/5* mutants, deficient in ABA biosynthesis, were similar to those of WT controls, at both 22°C and 35°C (fig. 3.2.4). This suggests that ABA does not constitute a major part of the UV-B-induced stomatal closure signalling pathway but may play a complementary role, or perhaps maintenance of stomatal closure after initial closure. This is supported by the results obtained by Tossi et al. (2009) who showed that ABA levels in maize almost doubled after 4 h UV-B irradiation. It would be interesting to investigate whether stomatal closure is maintained between 4 h and 24 h in the *nced3*/5 mutant. The role of ABA in NO accumulation is complex (Neill et al., 2008), but Wilson et al. (2009) showed that ABA is required for functional NO signalling in GCs. It was shown by Tossi et al. (2014) that NO accumulated after UV-B irradiation, and that the response is maintained for 24 h post-exposure. The NO pattern correlated with stomatal closure and the authors concluded NO

ultimately mediates stomatal closure. Here, the NO pattern also correlated with stomatal closure (fig. 3.2.2; 3.2.5a; 3.2.5b). Of special note with regards to high temperature signalling was that the lowest NO levels were observed in GCs treated at 35°C -UV-B, conditions which also resulted in greatest stomatal apertures. These experiments may therefore have identified a novel signalling component involved in high temperature-mediated stomatal opening. These data also suggest that one point of crosstalk between high temperature and UV-B signalling may involve NO, or the control of NO concentration or accumulation. It would be interesting to investigate the effects of added NO on high temperature-induced stomatal opening in the absence of UV-B. Future analyses could also include mutants deficient in potential NO generators, such as nitrate reductase to further investigate NO's involvement in the high temperature stomatal opening response. Figure 5.1 depicts our suggested model of the signalling pathway involving the components previously discussed.

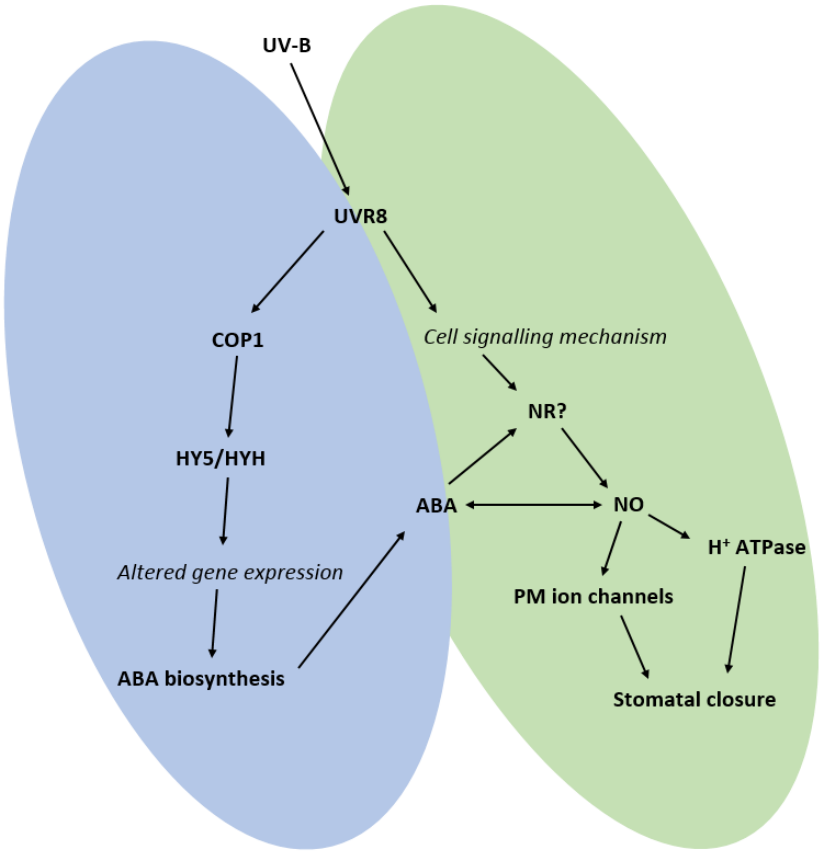


**Figure 5.1 Hypothetical UV-B signalling pathway leading to stomatal closure at high temperature.** UV-B is perceived by the UVR8 photoreceptor which acts to close stomata shortly after the onset of UV-B irradiation and maintain closure for up to 24 h (Tossi et al., 2014). The signalling components on the right-hand side (green) are suggested to act in the short-term closure response. This involves an unknown signalling mechanism resulting in altered intracellular NO levels. It is unclear how the NO is generated but may involve NR and/or ABA. The left hand side (blue) involves altered gene expression, which may be necessary for maintained stomatal closure. Tossi et al. (2014) showed the involvement of UVR8, COP1 and HY5/HYH in the UV-B-induced stomatal closure response at 22°C. Assuming that these components are also involved in the UV-B-induced stomatal closure response at high temperature, ABA biosynthesis may be the target for maintained stomatal closure.

Plants use sunlight to provide information about their surroundings in natural environments (Kami et al., 2010). Sunlight activates more than one photoreceptor so that plants can respond effectively to a dynamic environment (Casal, 2013). Often these photoreceptor pathways interact, providing plants with a more flexible system for light perception and ability to produce complex responses (Mazzella and Casal, 2001). Light has different quantities (fluence rates) as well as qualities (wavelength composition). This provides an extra level of specificity. The ability to close stomata in response to UV-B, thereby counteracting blue and red light-induced stomatal opening (Lurie, 1978; Chen et al., 2012), provides plants with the capacity to respond to changing light environments. Very little UV-B is transmitted through leaves to shaded areas and it has been suggested that plants perceive UV-B as a signal of direct sunlight (Hayes et al., 2017). Shade avoidance was inhibited by UV-B in the study by Hayes et al. (2014) by antagonising auxin and gibberellic acid signalling via inhibition of PIF4 and PIF5 abundance and activity. The authors speculated that this response leads to reduced allocation of resources towards growth, as growth is no longer necessary once neighbours are outgrown. A similar trade-off may be in operation with regard to stomatal opening. Reduced stomatal aperture lowers $CO_2$ uptake and carbon assimilation, resulting in less resources for growth. When plants are exposed to strong direct sunlight, photosynthesis is at capacity and keeping stomata wide open for $CO_2$ uptake risks excessive water loss. Due to the harmful nature of UV-B radiation, expression of protective pigments and antioxidants is elevated in plants exposed to UV-B radiation (Caldwell et al., 1983; Strid and Porra, 1992; Christie and Jenkins, 1996; Kalbin et al., 1997, 2001; Fraser et al., 2017). Stomatal closure in response to UV-B may therefore also allow reallocation of resources from stomatal opening to pigment and antioxidant production.

Plants are often exposed to high temperature and UV-B simultaneously, as both are associated with direct sunlight (Hayes et al., 2017). In addition to stomatal opening (Rogers et al., 1979; Hofstra and Hesketh, 1969; Feller, 2006), Crawford et al. (2012) showed that high temperature increases plant cooling capacity in Arabidopsis through increased transpiration despite producing fewer stomata. Here, stomatal opening was observed in Arabidopsis in response to 35$^{o}$C supporting the proposition that stomatal aperture is a likely means for evaporative cooling in this species. It would be interesting to measure water loss and plant biomass/carbon assimilation in the conditions used here to examine the effects of UV-B on WUE. It would be unfavourable for stomata to remain open at high temperatures, unless there was unlimited water available, as the risk for desiccation increases with water loss. Below the canopy of a rain forest, where water is abundant, stomata could potentially remain open without adverse effects. Convective cooling may be less effective in this environment due to due to reduced air movements within the canopy. Reduced sunlight penetration would remove both blue and UV-B signals, which acts to open and close stomata, respectively (Lurie, 1978; fig. 3.2.1a). Temperature-mediated control of stomatal aperture may therefore dominate control of leaf cooling in this environment. Limited UV-B exposure would additionally reduce the need for protective pigment production, enabling resources to be allocated elsewhere (e.g. growth).

High temperature-induced stomatal opening in an open, arid environment would expose plants to the risk of desiccation and death. In contrast to the rain forest habitat, plants in open environments receive plenty of sunlight. UV-B-induced stomatal closure may therefore have evolved to protect plants from harmful water stress that often occurs in high UV-B conditions. There is, however, likely to be an environment-specific trade-off for plants between cooling by evapotranspiration to prevent cellular damage by heat stress, and conserving water to ensure functional biochemistry and plant survival.

Global temperatures are predicted to rise in the future (fig. 1.3; Hartmann et al., 2013). There is extensive data on plant responses to different ambient temperatures, but our understanding of

thermosensory perception is only starting to develop (Delker et al., 2017). The data presented here provide further insight into the effects of temperature on plant physiology as well as signalling pathway crosstalk between temperature and light signalling. Plants are exposed to vast numbers of environmental signals simultaneously. Because so little is known about high temperature signalling, environmental cues which affect temperature signalling may provide potential targets for crop breeding for temperature tolerance. High temperature tolerance has proved difficult to breed for (Parent and Tardieu, 2012), but some progress has been made. For example, the NERICA-L-44 line of rice showed high seedling survival and better growth than other lines in a mutant screen (Bahuguna et al., 2015). Further research into the role of UV-B in controlling transpiration efficiency and water loss could be informative in regard to WUE in agriculture. Mutants with enhanced UV-B signalling (eg. *rup1/2* mutants; Gruber et al., 2010) could potentially reduce watering needs. Should such a scenario be possible, UV-B irradiation in glasshouses could improve plant robustness and aesthetic features (Fraser et al., 2017), increase plant antioxidant content (Kubis and Rybus-Zajac, 2008), as well as improve WUE, features which are all desirable for commercial crops.

## 5.2 Stomatal development

Long-term stomatal responses to changing environmental signals involve alterations in stomatal density and index. As discussed in chapter 4, stomatal index did not change in response to high temperature or UV-B in Col-0. This may suggest that the conditions used here (28$^{o}$C; 2.7 µmol $m^{-2}$ $s^{-1}$ UV-B) has little influence on initiation of the stomatal lineage. There was, however, a significant increase in stomatal index in response to UV-B at 28$^{o}$C in *uvr8-6*. Wargent et al. (2009) showed that stomatal index was not different between WT (*Ler*) and *uvr8-2* (*Ler* background) in the absence of UV-B. After treatment with diurnally varying UV-B irradiation of 0-5.2 µmol $m^{-2}$ $s^{-1}$ for 13 days, stomatal index increased in the WT but decreased in the mutant. UV-B fluence rate and timing of UV-B application could influence results. Alternatively, it may suggest that there is a temperature-dependent and UVR8-independent mechanism influencing stomatal development. However, the same result should arguably have been observed in the WT, but it may mean that UVR8 has an inhibitory role in stomatal development at 28$^{o}$C. Further replication is required to confirm this observation.

The stomatal density of Arabidopsis leaves decreased at high temperature while apertures increased (fig. 4.2.1; 3.2.1a). This may suggest that despite a reduction in stomatal density at high temperature, the increased apertures allow effective evapotranspiration and leaf cooling, as observed by Crawford et al. (2012). Considering that high temperatures rapidly increase stomatal aperture, if stomatal index was also increased, plants would risk excessive water loss. Alternatively, decreasing stomatal density may facilitate transpirational cooling, by improving air flow around stomata. Stomatal density decreased significantly in *uvr8-6* mutants in response to UV-B. No such reduction was seen in Col-0. This suggests that reduction in stomatal density in the conditions used here is UVR8-independent. The response seen in density but not in index further suggests that epidermal cell expansion is the mechanism affected by UV-B. Indeed, a UVR8-independent role for UV-B in regulating leaf area was reported by Wargent et al. (2009). High UV-B conditions often occur together with high temperatures and drought, resulting in a need for water preservation. Indeed, Glitz III et al. (2005) showed that a reduction in stomatal density, induced by UV-B irradiation, correlated with an increase in WUE in soybean. It would therefore be interesting to investigate the impact of UV-B-mediated changes in stomatal density on WUE. It is possible that UV-B-mediated changes in stomatal aperture and density combine to control plant WUE and leaf cooling which would be useful information for crop management.

In this study, technical limitations meant that relatively small plant growth areas could be subjected to UV-B exposure. To achieve a meaningful number of replicates for each experiment, plants were grown in close proximity. This may have resulted in some leaf shading, affecting results. Close neighbour proximity also meant that leaves chosen for analysis may not receive the direct UV-B and/or PAR dose intended. Altering light quality has striking effects on plant architecture (Franklin and Whitelam, 2005). Sufficient UV-B and PAR exposure could be achieved by increasing the growth area while ensuring uniform and sufficient UV-B exposure in future experiments. It would also be interesting to investigate whether the stomatal density response to UV-B is systemic. It has been shown that mature leaves of Arabidopsis relay information about their immediate environmental conditions to developing leaves, indicating systemic impact on stomatal development (Lake et al. 2002; Casson and Hetherington, 2014).

Growth conditions were also not controlled for $CO_2$. $CO_2$ has been shown to have profound effects on stomatal development. Woodward (1987) observed that an increase in global $CO_2$ of 60 µmol $m^{-2}$ $s^{-1}$ correlated with a 40% decrease in stomatal density of plant leaves collected over the last 200 years. It was later shown that $CO_2$ systemically influences stomatal density (Lake et al., 2002). The integration of UV-B, temperature and $CO_2$ signalling pathways in stomatal development would therefore be of interest.

## 5.3 Conclusions and further work

The results presented in this thesis underline significant effects of UV-B on both stomatal aperture and density. While stomata open in response to elevated temperatures, UV-B antagonises this response, causing stomatal closure, suggesting extensive crosstalk between high temperature and UV-B signalling. This supports the importance of investigating multiple signalling pathways simultaneously to understand plant physiology. With varying UV-B levels across the globe, an uncertainty of stratospheric ozone recovery and global warming, the effects of UV-B on plants exposed to high temperatures are central in our understanding of crop productivity, food security, and biome health. Results such as those obtained in this study could contribute towards improving agricultural practices, particularly to increase WUE, although further experiments are required. Further questions arising from this work include identification of the point of crosstalk between UV-B and high temperature; how UV-B ultimately closes stomata and further signalling components involved in the response. It would be interesting to use RNA sequencing to investigate whether any changes in gene transcripts occur during the treatments used here and if there are underlying alterations in gene expression following high temperature and/or UV-B exposure. The results obtained here, as well as those described in the literature suggest that NO is the mediator of stomatal closure. It would be interesting to look at NO generation and the closure response to different NO levels across a range of temperatures. Analysis of ion channel mutants could potentially elucidate how stomata are closed in response to UV-B. Due to limitations, the involvement of calcium signalling in stomatal responses to UV-B and high temperature were not explored. This provides a further avenue of investigation.

# Appendix A

## Supplementary information

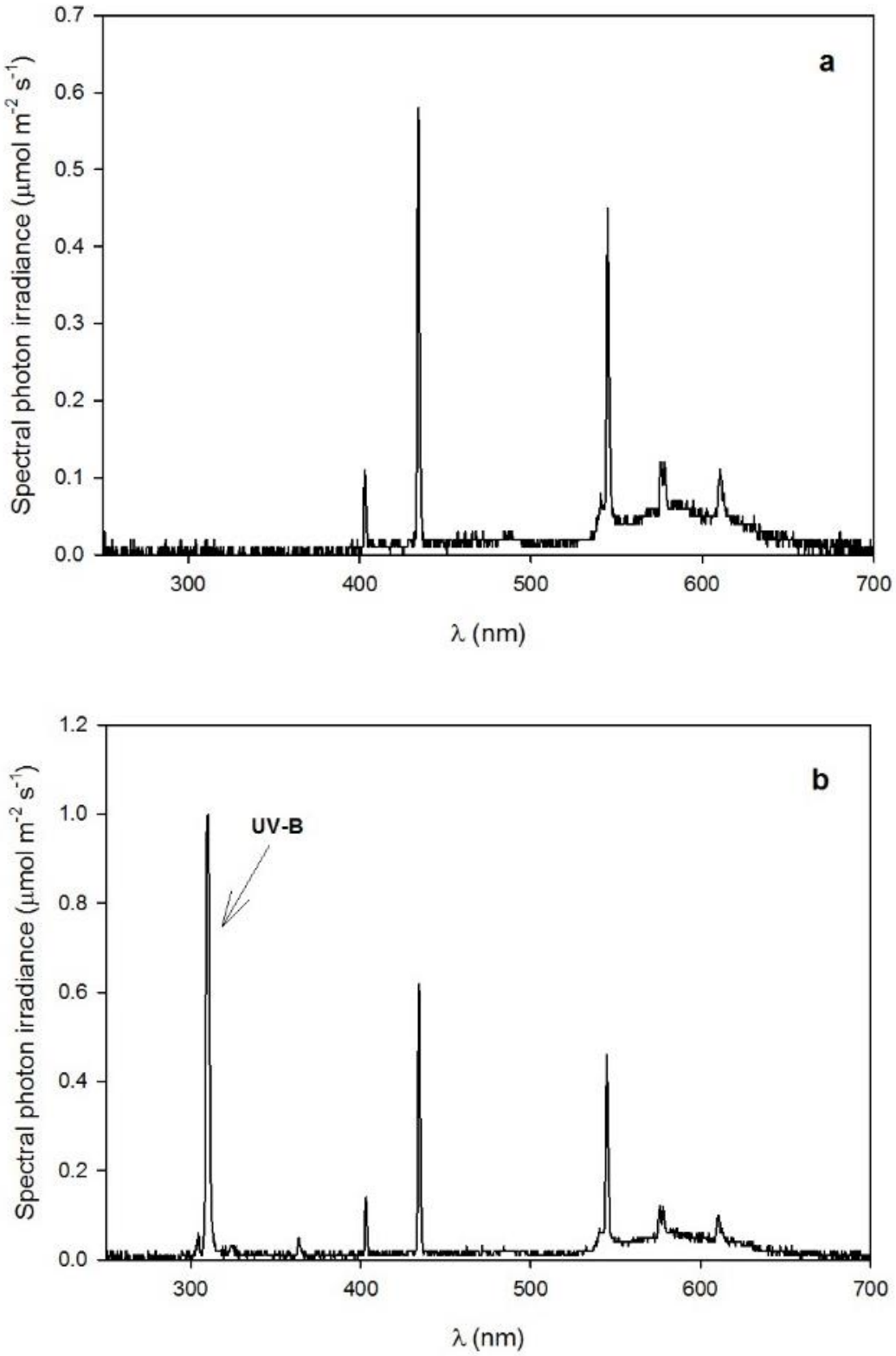


**Figure S1. Light spectra measured at sample height in bioassay tanks. (a)** White light -UV-B **(b)** White light +UV-B (2.7 µmol $m^{-2}$ $s^{-1}$).

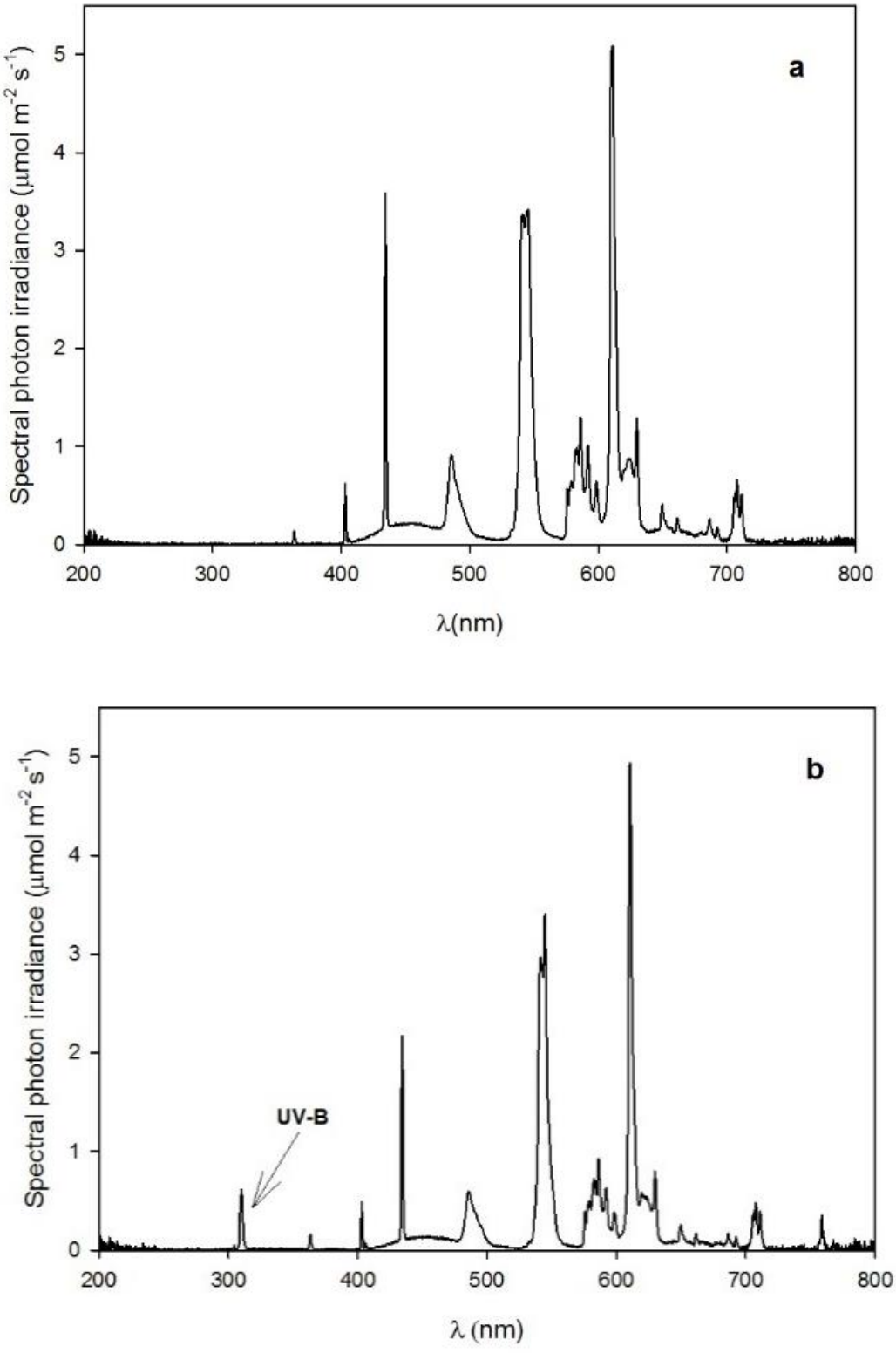


**Figure S2. Light spectra from plant growth cabinets. (a)** White light (100-115 µmol $m^{-2}$ $s^{-1}$) -UV-B. **(b)** White light (100-115 µmol $m^{-2}$ $s^{-1}$) +UV-B (2.7 µmol $m^{-2}$ $s^{-1}$).

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